MELATONIN IN ASSISTED REPRODUCTIVE TECHNOLOGY
THE MIART TRIAL

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Department of Obstetrics and Gynaecology

School of Clinical Sciences

Produced on archival quality paper

'…The women of Australia, are depending on you…'

Professor David L Healy

Dedication

To the great man who started it all. The man who inspired a naïve medical student to specialise in women's health and who fostered a budding interest in clinical research.

Thank you to the late and great,

Professor David Lindsay Healy

1948 - 2012

Without your guidance, belief and support, I may have become a neurologist.

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Table of Contents

Abstract

Through decades of development, the outcomes of assisted reproductive technology (ART), of which in-vitro fertilisation (IVF) is one form, have continued to improve. The field has progressed rapidly, from the dawn of ART, where the question was simply 'can we?' to now focussing on 'how can we do it better?' Naturally, with an increase in the accessibility, acceptability and uptake of ART treatments, and the advent of commercial ART, comes an increase in the demands on treatment to provide a 'successful' outcome - a live birth. During these years, significant improvements have been made to optimising patients for ART, ovarian stimulation protocols, culture media and luteal phase support. In the race to further improve outcomes, investigation into novel adjuvant therapies aimed at improving oocyte and embryo number and quality has begun, with a focus on immune system activity and oxygen scavengers.

Melatonin, a potent endogenous oxygen scavenger, has gained attention due to its ability to neutralise oxygen free-radicals in both intra- and extra-cellular environments and synergise the actions of other endogenous anti-oxidants. Melatonin is also a suicidal-terminal antioxidant, meaning that its metabolites are also antioxidants and therefore, melatonin does not promote oxidation under any circumstance. It has been conclusively shown that melatonin has a role in reproduction, however, its impact is still uncertain. Several investigators have suggested that it can improve oocyte and embryo number and quality and maybe even pregnancy rate after ART treatment. However, these studies have generally been small, unblinded, non-randomised and/or using a within-subjects' design. Despite this lack of adequate evidence, melatonin is currently used widely by infertility specialists, possibly because of pressure from patients or colleagues.

Through a pilot double-blind placebo-controlled randomised dose-finding clinical trial, this thesis aims to determine whether melatonin can improve clinical pregnancy rates after ART

through an improvement in oocyte and embryo number and quality. I further aspired to determine whether melatonin can alter blood flow to the reproductive organs, as this may explain a further mechanism of action of melatonin beyond its oxygen scavenging ability. Finally, I aimed to show that administration of melatonin, even at high doses given in the morning and evening, does not impair sleep patterns or sleep quality in these women.

While I show that exogenous oral administration of sustained-release melatonin does, indeed, result in a dose-dependent increase in both serum and follicular fluid concentrations of melatonin, this neither translates to an improvement in oocyte or embryo number or quality, nor an increase in clinical pregnancy rate. Furthermore, melatonin does not result in a difference in uterine artery or ovarian follicular blood flow. Interestingly, even when given in divided doses in the morning and evening, melatonin does not change daytime sleepiness, nor does it change night time sleep quality.

In conclusion, this work has shown that while exogenous oral administration of melatonin can result in supra-physiological follicular fluid concentrations and does not alter sleep patterns of patients, this does not appear to translate to a clinical benefit in the ART population. Although, this study is small and therefore lacks power to state this definitively, this suggests that the use of melatonin, in the doses tested, with the intention of improving ART outcomes should be discontinued until larger, similarly well-designed studies are performed. This work has contributed to the field of reproductive medicine and it is expected that it will result in a change in clinical practice.

Research output and Awards

PUBLICATIONS (RELEVANT TO THESIS)

1. **Fernando S**, Rombauts L. Melatonin: Shedding light on infertility? - a review of the recent literature. *Journal of Ovarian Research*, 2014. Vol 7, 98.

2. **Fernando S**, Osianlis T, Vollenhoven B, Wallace E, Rombauts L. A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol. *BMJ Open*, 2014. Vol 4, 8.

3. **Fernando S,** Rombauts L. Chapter 13: The use of melatonin to improve oocyte development. In: *How to Prepare the egg and embryo to maximize IVF success.* Eds. Kovacs GT, Rutherford AJ, Gardner DK. *Cambridge University Press,* Cambridge 2017.

PUBLICATIONS (DURING CANDIDATURE)

1. Rombauts L, Motteram C, Berkowitz E, **Fernando S**. Risk of placenta praevia is linked to endometrial thickness in a retrospective cohort of 4537 singleton assisted reproduction technology births. *Human Reproduction,* 2014. Vol. 29, 12

2. Rombauts L, McMaster R, Motteram C, **Fernando S**. Risk of ectopic pregnancy is linked to endometrial thickness in a retrospective cohort study of 8120 assisted reproduction technology cycles. *Human reproduction*, 2015

3. Pritchard N, **Fernando S**, Rombauts L. Hysteroscopy – an invasive diagnosis. In: The Endometrial Factor. Eds. Simon C, Giudice L. CRC Press, Abingdon 2017.

4. Kumar A, Nestel D, East C, Hay M, Lichtwark I, McLelland G, Bentley D, Hall H, **Fernando S**, Hobson S, Larmour L, Dekoninck P, Wallace EM. Embedding assessment in a simulation skills training program for medical and midwifery students: a pre and post intervention evaluation. Australian and New Zealand Journal of Obstetrics and Gynaecology, 2017

POSTER PRESENTATIONS

1. *'Melatonin in Assisted Reproductive Technology (MIART) –Oral melatonin treatment does not affect sleep – A double-blind randomized placebo-controlled trial'* – Society for Reproductive Investigation, Orlando, Florida, USA, 2017

2. *'The MIART Trial: Melatonin in Infertility and Assisted Reproductive Technology - a randomised controlled trial*' - Monash Institute of Medical Research Department Symposium, Melbourne, Australia, 2014

ORAL PRESENTATIONS

1. BFS Exchange Award winner presentation – '*Melatonin in assisted reproductive technology: A double-blind randomized placebo-controlled dose-finding clinical trial*' – British Fertility Society, Liverpool 2018
2. '*Melatonin in assisted reproductive technology: A double-blind randomized placebo-controlled dose-finding clinical trial*' – Fertility Society of Australia, Adelaide 2017
3. '*Melatonin in assisted reproductive technology: MIART trial. A pilot double-blind randomised placebo-controlled clinical trial*' – European Society of Human Reproduction and Embryology, Geneva, Switzerland 2017
4. '*Melatonin in assisted reproductive technology: MIART trial. A pilot double-blind randomised placebo-controlled clinical trial*' – Fertility Society of Australia, Adelaide, Australia 2017
5. *'The effect of melatonin on ultrasound markers of ovarian response'* – International Society of Ultrasound in Obstetrics and Gynaecology, Vienna, Austria, 2017 (presented by A/Prof Fabricio Costa on behalf of Dr Shavi Fernando)
6. '*The results of the MIART trial'* – 2017 Monash IVF Scientists' Day, Encore, Melbourne
7. '*Melatonin in Assisted Reproductive Technology – Preliminary results of the MIART trial*' – 2016 MHTP PhD Student Symposium, Hudson Institute, Melbourne
8. '*MIART: Melatonin in Assisted Reproductive Technology – An Update on progress*' – 2016 Monash IVF Scientists' Day, Luna Park, Melbourne
9. Departmental 3 Minute thesis Competition, The Ritchie Centre Hudson Institute of Medical Research
10. '*Melatonin in ART*' – MHTP Research Week 2015 invited speaker
11. *'Melatonin in assisted reproductive technology – The MIART trial'* – The Ritchie Centre Colloquium 2017

FUNDING AWARDED

1. National Health and Medical Research Council (NHMRC) Clinical Postgraduate Research Scholarship ($117,368 over three years, 2014-2016)
2. Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) Ella Macknight Memorial Scholarship ($50,000 over two years, 2015-2016)
3. Monash IVF Research Foundation Grant ($20,000, 2015)
4. Monash IVF Research Foundation Grant ($7,260, 2016)

FUNDING AWARDED BUT DECLINED

1. Monash University Faculty of Medicine, Nursing and Health Sciences, APA scholarship ($25000)
2. Faculty Postgraduate Excellence Award - awarded to top 8 candidates ($5000)
3. Senior Medical Staff Clinical Research Fellowship - Monash Health ($20000pa for three years)

AWARDS

1. Geoffrey Driscoll Medallist - Best Clinical Paper, British Fertility Society Exchange Award ($5000 travel grant) – Fertility Society of Australia, 2017
2. 1st Prize – Afternoon RANZCOG VRC Research symposium 2017 ($200)
3. 1st Prize – Departmental 3 Minute thesis Competition, The Ritchie Centre Hudson Institute of Medical Research, 2015
4. 3rd Prize - 'Melatonin in Assisted Reproductive Technology – Preliminary results of the MIART trial' – 2016 MHTP PhD Student Symposium, Hudson Institute, Melbourne

Declarations

GENERAL DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals and 2 unpublished publications. The core theme of the thesis is the effect of melatonin supplementation on IVF outcomes. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Hudson Institute of Medical Research and the Ritchie Centre under the supervision of Prof Euan Wallace and Prof Luk Rombauts.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Chapter 2 involved laboratory assistance provided by Dr Rebecca Lim, Dr Bryan Leaw, Ms Ruth Muljadi, Ms Siow Tang Chan, Ms Sinnee Lau and Dr Jean Tan. Dr Miranda Davies-Tuck provided statistical advice for Chapters 3 to 5.

In the case of Chapters 1 to 6 my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status**	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author Monash student Y/N**
1	Melatonin: Shedding light on infertility? - a review of the recent literature	Published	90%	1. Luk Rombauts, input into manuscript 10%	N
2	A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART):	Published	70% concept, development, writing of manuscript	1. Luk Rombauts 10%, input into manuscript and design 2. Euan Wallace 10%, input into manuscript and design 3. Tiki Osianlis 5%, input into manuscript 4. Beverley Vollenhoven 5%	N

	study protocol			input into manuscript	
3	Melatonin in Assisted reproductive technology: A pilot double-blind randomised placebo-controlled clinical trial	Submitted	80% concept, development, troubleshooting, recruitment, laboratory analysis, data analysis, manuscript preparation	1. Luk Rombauts 9%, design and concept, troubleshooting, input into manuscript 2. Euan Wallace 10% design and concept, troubleshooting, input into manuscript, laboratory advice 3. Melissa Wong, Nicole Hope, Beverley Volenhoven, Nicholas Lolatgis, Mark Lawrence, Anthony Lawrence, Philip Thomas, Kenneth Leong, Chris Russell 1% combined, recruitment, input into manuscript	N
4	The effect of melatonin on ultrasound markers of follicular development: a double-blind placebo-controlled randomised trial	Submitted	75% concept, development, troubleshooting, recruitment, data analysis, manuscript preparation	1. Fabricio Costa 10%, concept, data collection, input into manuscript 2. Nikki White 3%, data collection 3. Jennifer Hong 2%, data collection, input into manuscript 4. Luk Rombauts 5%, design and concept, troubleshooting, input into manuscript 5. Euan Wallace 4% design and concept, troubleshooting, input into manuscript 6. Melissa Wong, Nicole Hope, Beverley Volenhoven, Nicholas Lolatgis,	N

				Mark Lawrence, Anthony Lawrence, Philip Thomas, Kenneth Leong, Chris Russell 1% combined, recruitment, input into manuscript	
5	The impact of melatonin on the sleep patterns of women undergoing IVF: A double blind RCT.	Published	75% concept, development, troubleshooting, recruitment, data analysis, manuscript preparation	1. Sarah Biggs 10%, concept, design, data analysis, input into manuscript 2. Rosemary Horne 5%, concept, input into manuscript 3. Luk Rombauts 5%, design and concept, input into manuscript 4. Euan Wallace 4% design and concept, input into manuscript 5. Melissa Wong, Nicole Hope, Beverley Volenhoven, Nicholas Lolatgis, Mark Lawrence, Anthony Lawrence, Philip Thomas, Kenneth Leong, Chris Russell 1% combined, recruitment, input into manuscript	N
6	Discussion and Conclusions	Not for publication	100% writing of chapter	N/A	N/A

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:  **Date: 7/06/2018**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature:  **Date: 7/06/2018**

DECLARATION FOR THESIS CHAPTER ONE

Declaration by candidate

In the case of Chapter One, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Concept, development of figures, research, literature review and writing of manuscript	90%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Luk Rombauts	Input into manuscript	N/A

Candidate's signature:	[redacted]	Date: 07/06/2018

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Hudson Institute of Medical Research

Contributor	Signature	Date
Luk Rombauts	[redacted]	07/06/2018

DECLARATION FOR THESIS CHAPTER TWO

Declaration by candidate

In the case of Chapter Two, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Concept, development, writing of manuscript	80%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Luk Rombauts	Input into manuscript and design	N/A
Euan Wallace	Input into manuscript and design	N/A
Tiki Osianlis	Input into manuscript	N/A
Beverley Vollenhoven	Input into manuscript	N/A

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Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Hudson Institute of Medical Research

Contributor	Signature	Date
Luk Rombauts	[redacted]	07/06/2018
Euan Wallace	[redacted]	07/06/2018
Tiki Osianlis	[redacted]	07/06/2018
Beverley Vollenhoven	[redacted]	07/06/2018

DECLARATION FOR THESIS CHAPTER THREE

Declaration by candidate

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Concept, development, troubleshooting, recruitment, laboratory analysis, data analysis, manuscript preparation	85%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Luk Rombauts	design and concept, troubleshooting, input into manuscript	N/A
Euan Wallace	design and concept, troubleshooting, input into manuscript	N/A
Beverley Vollenhoven	recruitment, input into manuscript	N/A
Melissa Wong	recruitment, input into manuscript	N/A
Nicole Hope	recruitment, input into manuscript	N/A
Nicholas Lolatgis	recruitment, input into manuscript	N/A
Mark Lawrence	recruitment, input into manuscript	N/A
Anthony Lawrence	recruitment, input into manuscript	N/A
Philip Thomas	recruitment, input into manuscript	N/A
Kenneth Leong	recruitment, input into manuscript	N/A
Chris Russell	recruitment, input into manuscript	N/A

Candidate's signature:		Date: 07/06/2018

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Beverley Vollenhoven	[redacted]	**07/06/2018**
Melissa Wong	[redacted]	**07/06/2018**
Nicole Hope	[redacted]	**07/06/2018**
Nicholas Lolatgis	[redacted]	**07/06/2018**
Mark Lawrence	[redacted]	**07/06/2018**
Anthony Lawrence	[redacted]	**07/06/2018**
Philip Thomas	[redacted]	**07/06/2018**
Kenneth Leong	[redacted]	**07/06/2018**
Chris Russell	[redacted]	**07/06/2018**

DECLARATION FOR THESIS CHAPTER FOUR

Declaration by candidate

In the case of Chapter Four, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Concept, development, troubleshooting, recruitment, data analysis, manuscript preparation	85%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Luk Rombauts	design and concept, troubleshooting, input into manuscript	N/A
Euan Wallace	design and concept, troubleshooting, input into manuscript	N/A
Fabricio da Silva Costa	concept, data collection, input into manuscript	N/A
Nikki White	data collection, input into manuscript	N/A
Jennifer Hong	data collection, input into manuscript	N/A
Beverley Vollenhoven	recruitment, input into manuscript	N/A
Melissa Wong	recruitment, input into manuscript	N/A
Nicole Hope	recruitment, input into manuscript	N/A
Nicholas Lolatgis	recruitment, input into manuscript	N/A
Mark Lawrence	recruitment, input into manuscript	N/A
Anthony Lawrence	recruitment, input into manuscript	N/A
Philip Thomas	recruitment, input into manuscript	N/A
Kenneth Leong	recruitment, input into manuscript	N/A
Chris Russell	recruitment, input into manuscript	N/A

Candidate's signature:		Date: 07/06/2018

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
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Fabricio da Silva Costa	[redacted]	**07/06/2018**
Nikki White	[redacted]	**07/06/2018**
Jennifer Hong	[redacted]	**07/06/2018**
Beverley Vollenhoven	[redacted]	**07/06/2018**
Melissa Wong	[redacted]	**07/06/2018**
Nicole Hope	[redacted]	**07/06/2018**
Nicholas Lolatgis	[redacted]	**07/06/2018**
Mark Lawrence	[redacted]	**07/06/2018**
Anthony Lawrence	[redacted]	**07/06/2018**
Philip Thomas	[redacted]	**07/06/2018**
Kenneth Leong	[redacted]	**07/06/2018**
Chris Russell	[redacted]	**07/06/2018**

DECLARATION FOR THESIS CHAPTER FIVE

Declaration by candidate

In the case of Chapter Five, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Concept, development, troubleshooting, recruitment, data analysis, manuscript preparation	85%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Luk Rombauts	design and concept, troubleshooting, input into manuscript	N/A
Euan Wallace	design and concept, troubleshooting, input into manuscript	N/A
Sarah Biggs	concept, data collection, input into manuscript	N/A
Rosemary Horne	data collection, input into manuscript	N/A
Beverley Vollenhoven	recruitment, input into manuscript	N/A
Melissa Wong	recruitment, input into manuscript	N/A
Nicole Hope	recruitment, input into manuscript	N/A
Nicholas Lolatgis	recruitment, input into manuscript	N/A
Mark Lawrence	recruitment, input into manuscript	N/A
Anthony Lawrence	recruitment, input into manuscript	N/A
Philip Thomas	recruitment, input into manuscript	N/A
Kenneth Leong	recruitment, input into manuscript	N/A
Chris Russell	recruitment, input into manuscript	N/A

Candidate's signature:		Date: 07/06/2018

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
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Location(s)	**Hudson Institute of Medical Research**

Contributor	**Signature**	**Date**
Luk Rombauts	[redacted]	**07/06/2018**
Euan Wallace	[redacted]	**07/06/2018**
Sarah Biggs	[redacted]	**07/06/2018**
Rosemary Horne	[redacted]	**07/06/2018**
Beverley Vollenhoven	[redacted]	**07/06/2018**
Melissa Wong	[redacted]	**07/06/2018**
Nicole Hope	[redacted]	**07/06/2018**
Nicholas Lolatgis	[redacted]	**07/06/2018**
Mark Lawrence	[redacted]	**07/06/2018**
Anthony Lawrence	[redacted]	**07/06/2018**
Philip Thomas	[redacted]	**07/06/2018**
Kenneth Leong	[redacted]	**07/06/2018**
Chris Russell	[redacted]	**07/06/2018**

DECLARATION FOR THESIS CHAPTER SIX

Declaration by candidate

In the case of Chapter One, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Writing of chapter	100%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
N/A	N/A	N/A

Candidate's signature:	[redacted]	Date: 07/06/2018

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A project of this size could not have been undertaken without significant input from other experienced scientists. In particular, Dr Rebecca Lim, Dr Bryan Leaw, Dr Siow Tang Chan, Sinnee Lau, Ruth Muljadi and Dr Jean Tan. Thank you for your support and patience, teaching and guidance in the laboratory.

I would also like to express my gratitude to Monash IVF, for welcoming me into their eminent research department. Thank you also to the incredibly hardworking research team at Monash IVF, in particular Vivien Maclachlan, Deborah Deguingand and Caroline Motteram. Your humour, positivity and work ethic are something that I admire and am eternally grateful for. I am indebted to all of the welcoming staff at Monash IVF, particularly the infertility fellows Dr Sameer Jatkar and Dr Devashana Gupta, IVF nurses, embryologists and theatre staff. Thank you for keeping me sane and providing me with stimulating conversation when waiting for samples.

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To Ella, our little bundle of energy. Thank you for being the delightful baby you are. Thank you for your understanding of the stresses involved in full-time research and of course, thank you for sleeping so well! Finally, none of this would have been possible without the love, understanding, patience and support of my dear wife, Sharmayne. I cannot thank you enough (and never expect to be able to) for delaying your career progression to bring Ella into the world. I know how difficult a sacrifice this was for you, and I am immensely grateful. You are the most amazing mother and wife, and I am thankful every day that I am with you.

List of Figures

List of Tables

Chapter One

Melatonin in assisted reproductive technologies

1.1 A SUMMARY TO BEGIN AND THESIS STRUCTURE

This thesis aims to determine whether a commonly prescribed antioxidant, melatonin, can improve the outcomes for infertile patients undergoing in-vitro fertilisation (IVF).

Since its inception in the 1970s, in-vitro fertilisation (IVF) and other assisted reproductive technologies (ART) have expanded globally, with almost one in twenty Australian births resulting from some form of assisted reproduction (Harris, et al., 2016). Despite significant improvements in technology, embryology and early pregnancy care, the successful live birth rate has remained relatively stable over the preceding decade (Harris, et al., 2016). In Australia, the recent commercialisation of IVF clinics and an increase in reliance on ART techniques, in part as a result of the aging maternal population, has seen an increase in pressure to improve outcomes following ART treatment. Many clinicians, and indeed, patients, are seeking novel adjuvant therapies designed to significantly improve their chances of taking home a baby.

Fertility specialists are increasingly prescribing treatments with little or no quality evidence of efficacy or safety under the impression that these therapies will improve the chances of success after ART treatment without causing harm. Adjuvants currently being used without sound evidence to support them range from seemingly harmless vitamin C and fish oil to more invasive immunoglobulin infusions and acupuncture. Certainly, if safety has been proven, clinicians justify treatment under the belief that 'if it does no harm, then why not try it?' Unfortunately, in many cases of adjuvant therapy, adequate data supporting efficacy and safety have not been elucidated, and specialists and patients turn to these treatments as 'just another thing to try'. This can be harmful to patients, not only because of the risks and financial toxicity that may be involved, but also because of the provision of 'false hope'. This overestimation of the chances of success with these adjuvant therapies can result in

significant disappointment amongst the infertile population and contribute to complaints received by ART clinics.

One such adjuvant treatment that is currently used by infertility specialists is melatonin. Melatonin, an indoleamine secreted by the pineal gland in response to darkness, is a natural regulator of mammalian circadian rhythm. It is currently approved for use in Australia as a treatment for insomnia in selected populations. It is also a very potent oxygen scavenger, with metabolites that also act as anti-oxidants. Its ability to potentiate the actions of other endogenous antioxidants and its relatively easy commercial availability has made it a popular 'off-label' addition to standard therapy for many IVF clinicians. The theory behind its benefit and available evidence supporting this is thoroughly reviewed in *Chapter One* and forms the foundation for the Melatonin in Assisted Reproductive Technology (MIART) Trial.

The design of the MIART trial is described in detail in *Chapter Two* and includes a description of the selected population, the trial protocol and outcome measures, in particular, the primary outcome – the effects of melatonin on clinical pregnancy rates in ART patients. This primary outcome is investigated and presented in *Chapter Three*, together with other important clinical outcomes including oocyte and embryo number and quality, miscarriage rate and adverse event rates. Based on the hypothesis that melatonin does improve outcomes of ART, I also aimed to determine how melatonin might influence such outcomes. Because of the known effect that melatonin has on some vascular beds, I aimed to determine whether melatonin has an impact on ovarian and uterine blood flow during ART. The results of this investigation are presented in *Chapter Four*, together with the effect of melatonin on other ultrasound markers of ovarian response to stimulation including follicle size and number. If melatonin does improve success rates after fertility treatment, it would then be important to show that it does not have any negative effects on the population studied. One effect of melatonin is its potential ability to impair sleep quality or increase daytime sleepiness, particularly when given in the morning and evening. This was investigated using

both subjective and objective measures of sleep and is presented in *Chapter Five*. Finally, *Chapter Six* discusses and summarises the findings and limitations of this work and provides an insight into future research which may improve the outcomes of future generations of infertile patients.

1.2 AIMS

The overall aim of this thesis is to determine the effect, if any, that oral melatonin has on ART outcomes when given during ovarian stimulation. This will be elucidated using the following three aims:

1. To determine whether melatonin can improve oocyte and embryo number and quality and clinical pregnancy rate when given during ART treatment
2. To determine whether melatonin can improve ovarian and uterine blood flow and follicular markers of ovarian response on ultrasound when given during ART treatment
3. To determine whether melatonin affects night-time sleep quality and quantity or daytime sleepiness when given during ART treatment.

These aims are addressed in published or submitted works which have been included as the major component of this thesis.

1.3 BACKGROUND

Both in animal and human studies, melatonin has shown promise in its ability to promote fertility. Prior to embarking on this investigation, the usefulness of adjuvant therapies in infertility treatments has been called into question. The pressures on IVF clinics to improve IVF success rates, the advent of the internet and 'finger-tip' access to questionable advice for patients has resulted in a rise in the popularity of adjuvant therapies in IVF (Segev, et al., 2010). An in-depth review of recent literature surrounding melatonin and its relevance to the reproductive system and infertility treatments is described below.

1.4 MELATONIN: SHEDDING LIGHT ON INFERTILITY? – A REVIEW OF THE RECENT LITERATURE

Fernando and Rombauts *Journal of Ovarian Research* 2014, **7**:98
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Melatonin: shedding light on infertility? - a review of the recent literature

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Abstract

In recent years, the negative impact of oxidative stress on fertility has become widely recognised. Several studies have demonstrated its negative effect on the number and quality of retrieved oocytes and embryos following in-vitro fertilisation (IVF). Melatonin, a pineal hormone that regulates circadian rhythms, has also been shown to exhibit unique oxygen scavenging abilities. Some studies have suggested a role for melatonin in gamete biology. Clinical studies also suggest that melatonin supplementation in IVF may lead to better pregnancy rates. Here we present a critical review and summary of the current literature and provide suggestions for future well designed clinical trials.

Keywords: Melatonin, Oxidative stress, Oxygen scavenger, Infertility, In-vitro fertilisation

Introduction

Over the last 35 years, infertility treatment has become more acceptable and with improvements in technology the pressure for improved success rates has mounted. This trend is perpetuated by a perceived ability to delay and yet successfully achieve pregnancy through assisted reproductive technologies (ART) [1]. Technological advancement and societal expectations therefore mandate continual improvement in in-vitro fertilisation (IVF) success rates, inspiring research into novel adjuvant therapies designed to improve IVF outcomes. More recently, it has been discovered that an imbalance of reactive oxygen species, or 'oxidative stress', can have a negative impact on the success of infertility treatments, and furthermore, investigators have begun addressing potential mechanisms of preventing these effects with the use of novel oxygen scavengers such as melatonin. It may be that these agents have a positive effect on pregnancy success rates following IVF treatment. We present a summary of the most recent work investigating melatonin and its affect on oxidative stress, with a focus on the reproductive system and the treatment of infertility.

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Melatonin

Melatonin: synthesis and degradation

Melatonin (N-acetyl-5-methoxytryptamine) was first isolated in 1958 as a neuro-hormone mainly synthesised and secreted from the pineal gland [2]. Since its discovery, further investigation has revealed that it is also produced by several other organs. It has been found in the gastrointestinal tract [3], brain [4], eye [5], lungs [6], skin [7], kidney [8], liver [9], thyroid, thymus, pancreas [10], immune system [11] and reproductive system [12]. Melatonin is an indoleamine, which is synthesised from the essential amino acid, tryptophan [13]. Its production is dependent on ambient illumination, with release being suppressed by light. Hence, endogenous levels in plasma begin to increase between 1800 and 2000 hrs and peak between midnight and 0500 hrs with levels before 0900 hrs being five times higher than those after 1100 hrs [14]. This diurnal variation can make comparative studies challenging.

In an investigation of the pharmacokinetics of exogenous orally administered melatonin, Waldhauser and associates found that the increase in serum levels after oral administration of melatonin is rapid (60–150 minutes), as is its excretion [15]. It does not accumulate in the blood, with repeat dosing simply resulting in peak levels being maintained for longer [15]. Melatonin is hepatically metabolised and renally excreted [16]. Hence, melatonin has a short half-life and both melatonin and its metabolites can be measured in serum, urine and saliva [17,18].

Actions and safety of melatonin

Classical actions

Melatonin has been identified as a key factor in the regulation of circadian rhythms and the sleep-wake cycle [18]. Long exposure to artificial lighting leads to a reduction in endogenous melatonin exposure [19]. Melatonin is thus associated with sleep disturbances including insomnia, and much of the literature is focused in this area [20-22]. It also appears to regulate reproductive seasonal variation in many animal species [18,23-25]. However, despite a daily circadian rhythm being demonstrated in uterine artery blood flow [26], seasonal breeding does not apply to primates [27], raising questions as to what other roles it may serve in humans.

Actions as an oxygen scavenger

Free oxygen radicals are created when oxygen is utilised in metabolic processes. These radicals contain 'free' valence electrons, making them highly reactive, capable of causing injury to cells [28]. The term 'reactive oxygen species' (ROS), not only includes free radicals but also stable non-radical molecules which are capable of causing oxidation, such as hydrogen peroxide (H_2O_2) [29]. While ROS are necessary for essential physiological processes, an over-abundance can result in cellular damage, commonly referred to as 'oxidative stress' [30]. Anti-oxidative agents (oxygen-scavengers) are present endogenously but can also be administered exogenously. They reduce free radicals by donating electrons to stabilise them [31].

Recently, it has been discovered that melatonin has important oxygen-scavenging properties [32-34]. Compared with other oxygen scavengers, melatonin is of particular interest because it has several qualities distinguishing and rendering it superior to classical anti-oxidative agents. For example, it has anti-oxidative effects through its receptors, MT1 and MT2 [35], but also as a direct free radical scavenger [36,37]. It has binding sites within the nucleus [13,38], and is amphiphilic, allowing it to cross cell membranes freely [36,39]. But one of its most unique characteristics is that, unlike classical anti-oxidants, melatonin is a suicidal terminal anti-oxidant. It does not promote oxidation under any circumstances and its metabolites are also capable of acting as anti-oxidants in a 'scavenging cascade reaction' without themselves becoming oxidative [37,40-42]. Importantly, melatonin also enhances the activity of other endogenous anti-oxidants including glutathione peroxidase and superoxide dismutase (Figure 1) [43-47].

These unique characteristics have made melatonin the subject of investigation into medical conditions in which oxidative stress has been implicated, including diabetes, glaucoma, irritable bowel syndrome and even in curtailing the side effects of chemo- and radiotherapy [48,49]. Melatonin has been shown to suppress tumour growth factors and angiogenesis, suggesting a possible role for melatonin in prevention of cancer growth [9,50,51]. Furthermore, melatonin has been shown to have anti-inflammatory and DNA stabilising actions in the lung [6,52], skin and intestine [53-56] and can help reduce chronic pelvic pain in women with endometriosis [57].

Importance of melatonin in reproduction

In humans, the only data on cyclical melatonin changes comes from women undergoing ovarian stimulation. Levels of melatonin reach a nadir in the preovulatory phase and peak in the luteal phase (Figure 2) [58-60]. This suggests that melatonin has variable effects dependent on the menstrual phase.

It is also well known that shift-workers are more likely than daytime workers to experience circadian disruption and longer menstrual cycles, more menorrhagia and dysmenorrhoea [61,62]. These results are corroborated by a very large cohort study, which also found that duration of shiftwork was modestly associated with menstrual cycle irregularity [63]. A Japanese study found that melatonin levels varied significantly between night and day shift workers, while LH and FSH levels did not, suggesting that the menstrual irregularity associated with shiftwork could be explained by melatonin fluctuations [64].

These findings are in line with central effects on the hypothalamic pituitary axis, being capable of modifying the release of gonadotrophins and GnRH [65]. In fact, in very high doses, when combined with progesterone, melatonin has the ability to suppress ovulation in humans, possibly by interfering with LH release [66]. This may represent an evolutionary remnant with inhibition of ovulation during darker months designed to prevent the birth of offspring when resources are less abundant.

Interestingly, melatonin receptors have been found on granulosa cells, indicating that this may be an additional site of melatonin activity [65,67,68]. Indeed, when given systemically in cats, melatonin appears to accumulate preferentially in the ovaries compared with other organs [69] and higher concentrations of melatonin are found in preovulatory follicular fluid than in serum [36,70,71]. A human study by Nakamura et al. [72] found that larger preovulatory follicles had higher concentrations of follicular fluid melatonin than smaller immature follicles. This is the only study that has addressed follicular fluid differences within the same patient, and indicates that follicular fluid from mature follicles have higher anti-oxidant capacity than smaller follicles, implying a role for melatonin in oocyte maturation. However, it is as yet unclear whether this is a cause or consequence.

Adding further credence to the role of melatonin in reproduction, melatonin requirements appear to increase during pregnancy [73], and researchers have begun to assess its role as a potential therapy in pre-eclampsia and

Fernando and Rombauts *Journal of Ovarian Research* 2014, **7**:98
http://www.ovarianresearch.com/content/7/1/98

neonatal neurological morbidity [74,75]. Recent investigations have shown that in ovine models, intrauterine infusion of melatonin results in an increase in umbilical artery blood flow and higher fetal-placental weight ratio. Importantly, intrauterine infusion of a melatonin receptor antagonist decreased fetal aortic blood flow relative to fetal weight, suggesting that activation of melatonin receptors may be the mechanism behind the apparent increase in fetal blood flow after oral melatonin supplementation [76]. Melatonin has also been shown to reduce the neurological effects of oxidative stress-induced fetal brain injury in rats and sheep [77,78]. These findings support a beneficial role of melatonin in the treatment and/or prevention of placental dysfunction, which may even extend to the treatment of pre-eclampsia and neurological damage in preterm and growth restricted neonates [4,78].

Because melatonin levels naturally decrease with age [79,80], some investigators have found that supplementation

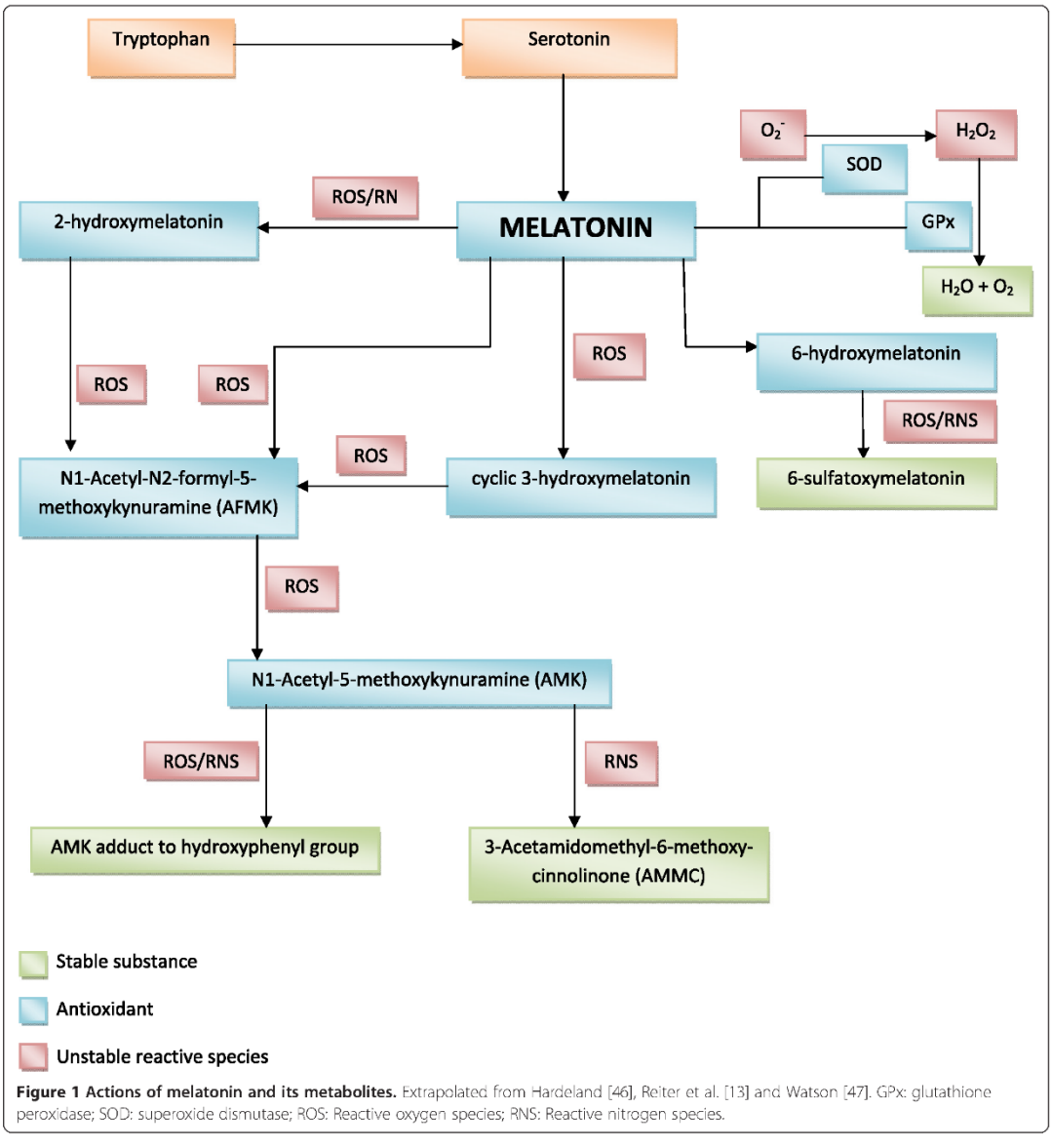

Figure 1 Actions of melatonin and its metabolites. Extrapolated from Hardeland [46], Reiter et al. [13] and Watson [47]. GPx: glutathione peroxidase; SOD: superoxide dismutase; ROS: Reactive oxygen species; RNS: Reactive nitrogen species.

Fernando and Rombauts *Journal of Ovarian Research* 2014, **7**:98
http://www.ovarianresearch.com/content/7/1/98

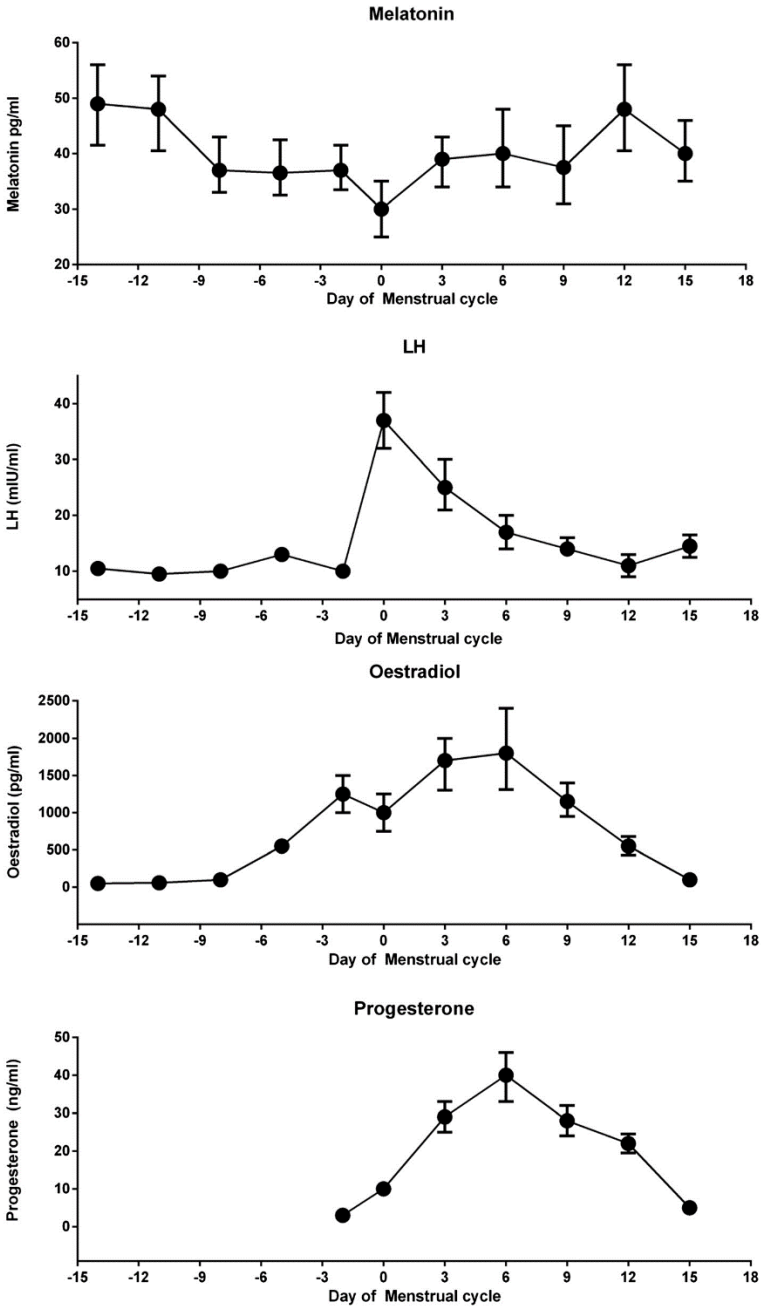

Figure 2 Relative concentrations of plasma melatonin, LH, estradiol and progesterone in hMG/hCG treated cycles. Adapted with permission from Tang et al. [59]. LH: Luteinising hormone.

may also have a role in the climacteric [81]. Melatonin also appears to have a role in the prevention of postmenopausal bone loss, with effects being exerted via inhibition of oxidative stress, induction of osteoblastogenesis and inhibition of osteoclastogenesis [82]. These findings and evidence from a small randomised controlled trial suggests that melatonin may be useful in the treatment of perimenopausal and menopausal symptoms and sequelae [83,84].

The positive implications of higher melatonin levels on the human menstrual cycle, fertility and pregnancy are therefore well documented, with varying levels of evidence [32-34,85]. Nevertheless, it appears clear that melatonin serves a purpose in the human reproductive system, with many of its observed effects likely to be related to its ability to dampen the effects of oxidative stress on the reproductive system.

Safety

Given the potential clinical benefits of melatonin it is equally important to assess its potential for harm, particularly when considering treatment in infertile or pregnant populations. It is reassuring that melatonin has a remarkably benign safety profile in both animal and human studies, with no teratogenic effects [86-88]. Furthermore, melatonin does not have significant sedative effects and is not associated with hepato-nephrotoxicity [89] even at supraphysiological doses (5 - 20 mg/day) for prolonged periods of administration (up to 12 weeks) in both adults and children [66,90-92]. While non-toxic, it has been suggested that melatonin can adversely affect autoimmune conditions, particularly rheumatoid arthritis [93], through its immuno-stimulatory actions. There have also been two case-reports of melatonin being associated with autoimmune hepatitis [94,95], and a suggestion that it may be implicated in multiple sclerosis through T-cell activation [96]. Though these effects are associative and follow biological plausibility, causality has not yet been proven [97]. Despite this, it is recommended to avoid the use of melatonin in those with autoimmune conditions.

Infertility treatment

The importance of oxidative stress in assisted reproductive technology (ART)

The relevance of oxidative stress in ART has gained increasing attention in recent literature, in particular with regards to IVF. IVF can result in exposure of oocytes and embryos to high levels of superoxide free radicals, which begins prior to oocyte retrieval [98]. Ovarian stimulation protocols are associated with significant changes to the *in-vivo* follicular environment, altering endogenous levels of oxygen scavengers [99]. Furthermore, in-vitro, these oocytes are no longer protected by antioxidant-rich follicular fluid, leaving them more susceptible to oxidative damage [100-102]. They may also be exposed to high oxygen concentrations in incubators and during handling throughout the IVF process, with higher concentrations of oxygen being associated with more ROS, and a positive effect of melatonin being more marked in oocytes exposed to higher oxygen tensions [103]. This oxidative stress modifies the quality of oocytes and embryos, decreasing the fertilisation rate and the success of the infertility treatment [104-106].

Investigators have found an inverse relationship between follicular fluid levels of ROS and success of ART, and these differences do not seem to be related to the cause of infertility. Bedaiwy et al. sequentially analysed the follicular fluid from 138 patients undergoing intracytoplasmic sperm injection ICSI [107]. They found that cycles that resulted in pregnancy were associated with a significantly higher total antioxidant capacity (a measure of the summative effect of antioxidants in the serum [108]) and significantly lower level of ROS [107] but the sample sizes were relatively small. This evidence suggests that intra-follicular oxidative stress may have a significant impact on IVF success rates.

While reactive oxygen species are required for sperm capacitation events [109,110], an imbalance of ROS has been implicated as a factor in reducing the quality and function of sperm [111], with most protection from these effects being afforded by the enzymatic antioxidant, superoxide dismutase [112-116].

One reason for the increased susceptibility of sperm to oxidative stress is the abundance of oxidative targets such as polyunsaturated fatty acids in the plasma membrane of sperm required for fusion with the oocyte and fertilisation [117]. Furthermore, it has been found that DNA fragmentation resulting from both *in-vivo* and in-vitro oxidative stress is a major contributor to poor sperm quality and function, and that antioxidative therapies may hold promise in attenuating these effects [118]. As might be expected, such DNA damage has been shown to have a negative impact on fertilisation, blastocyst development [119], and miscarriage rates and pregnancy outcome [120].

The recognition of the association between exposure of gametes and embryos to oxidative stress and a reduction in the success rates of IVF has led investigators to assess whether these adverse effects can either be prevented or reversed, with emphasis being placed on the adjuvant use of oxygen scavengers including melatonin.

The role of melatonin in assisted reproductive technology

Oxidative stress occurs at many levels during the treatment of infertility. Interventional studies have begun recently, with an emphasis on oral supplementation of melatonin during the ovarian stimulation phase of the IVF cycle and its effects on gamete and embryo quality. Clinical studies assessing the use of melatonin in IVF are

summarised in Table 1 and discussed in more detail below.

Effects of melatonin on oocyte quality

Melatonin is an effective mitigator of mitochondrial DNA damage [121], likely as a result of an increase in electron transport efficiency within mitochondria, thus preventing the formation of ROS [122]. In some situations melatonin may be even more effective at performing this function than specific mitochondrial antioxidants [123], and this particular characteristic may have relevance to its use in the treatment of infertility and the improvement of oocyte quality and maturity.

Oocyte quality begins to deteriorate immediately following ovulation, a process thought to be inflammatory [124] and through its production of cytokines and proteases is associated with an increase in ROS which can inhibit oocyte maturation [13,125,126]. A very recent murine study found that oxidative stress in oocytes may begin as early as 8 hours after ovulation, rising exponentially thereafter. This study also found that in-vitro addition of 1 mM of melatonin to oocyte culture media significantly ameliorated these time-dependent effects, resulting in 54% of fertilise oocytes reaching the blastocyst stage in the presence of melatonin compared with 29% in the controls [127]. This study not only showed that an imbalance of ROS is an important cause of impaired oocyte quality in-vitro, but also that the addition of melatonin could reverse these effects.

The follicular environment is naturally protective against oxidative damage to the oocyte [128]. To illustrate this, Tamura et al. sampled follicular fluid at oocyte retrieval and measured intrafollicular concentrations of melatonin and the oxidative stress marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG). Melatonin concentrations were directly proportional to follicular growth and, as expected, inversely correlated with 8-OHdG levels.

Kang et al. [129] investigated in-vitro porcine oocyte media supplemented with and without melatonin. They found a significantly lower level of ROS and a greater proportion of MII (mature) oocytes in the melatonin group but without an increase in cleavage frequency or blastocyst cell number. Tamura et al. [125] incubated mouse germinal vesicles exposed to H_2O_2 with several different concentrations of melatonin. After 12 hours, a positive dose–response relationship was found between increasing amounts of melatonin and the number of mature oocytes. These results strongly suggest that melatonin supplementation in-vitro is associated with a reduction in oxidative stress and improved oocyte maturation.

The literature is conflicting, however, with other animal studies finding an optimal melatonin range of 10^{-6} to 10^{-9} M in in-vitro maturation media, with both higher and lower doses having negative effects [130,131]. These findings are in agreement with human studies which have demonstrated that lower concentrations of melatonin in culture media improved nuclear maturation rate of immature MI oocytes [132], implantation rate and an insignificant increase in clinical pregnancy rate [133] with an optimal threshold of 10^{-5} M to 10^{-9} M. Both studies agreed that higher concentrations worsened outcomes. Although there is significant evidence to support a role for melatonin in oocyte maturation in-vitro, further investigation is warranted to confirm the optimal effective dose.

A recent review concluded that oral administration of melatonin reduces intrafollicular oxidative damage and increases fertilisation rates [36]. Unfortunately, most studies addressing the use of melatonin in infertility treatment have been conducted with patients as their own controls ('before and after' comparison) [36,125,134]. In the absence of proper control or placebo groups, it must be assumed that any beneficial effects thus observed are explained by the phenomenon of regression toward the mean [135,136].

Other human studies have been promising, but unfortunately, have also been challenged by design limitations. Eryilmaz et al. [137] performed an unblinded randomise controlled trial assessing melatonin supplementation in women with sleep disturbances undergoing IVF. The investigators randomise 30 patients to receive 3 mg nocte of oral melatonin from day 3–5 of their cycle up until administration of the human chorionic gonadotrophin (HCG) trigger. Controls received no additional treatment. They found a significantly increased number of oocytes, increased number of metaphase II oocytes and increased percentage of Grade 1 embryos (69.3% vs 44.8%, $p < 0.05$). The authors did not mention controlling or accounting for concurrent adjuvant treatments, nor did they account for the number of previous failed IVF cycles. In addition, their patients had a mean duration of infertility of 6–7 years and the aetiology of infertility was not considered.

Despite its limitations, these findings were in keeping with another larger unblinded randomised trial looking specifically at the effect of melatonin on IVF outcomes. Eighty women were randomised to receiving melatonin 3 mg/day or no treatment from the commencement of GnRH agonist administration. The percentage of mature oocytes was higher in the melatonin group ($p < 0.05$) as was the proportion of high quality embryos, however, an increase in clinical pregnancy rate did not reach statistical significance [138]. Additionally, patients with cancelled cycles were not included in the analysis making these findings susceptible to attrition bias.

One drawback of the studies already discussed is the lack of a placebo control. Others have overcome the challenge of recruiting patients for a placebo-controlled

Fernando and Rombauts *Journal of Ovarian Research* 2014, **7**:98
http://www.ovarianresearch.com/content/7/1/98

Table 1 Summary of human studies assessing the use of melatonin in IVF

Study	Design	NICE Level of evidence	Sample size	Intervention	Control	Outcomes
Melatonin alone						
Tamura et al. 2012 [36]	Uncontrolled before - after study	2^-	9	3 mg melatonin po from day 5 of menstrual cycle to oocyte collection (n = 9)	Previous cycle without melatonin (n = 9)	Higher rate of good embryos in melatonin cycle (65% vs 27%)*
Tamura et al. 2008 [125]	Prospective cohort	2^+	115	3 mg melatonin po from day 5 to oocyte collection (n = 56)	No melatonin (n = 59)	No difference in fertilisation or clinical pregnancy rate
Tamura et al. 2008 [125]	Uncontrolled before - after study	2^-	112	3 mg melatonin po from day 5 to oocyte collection (n = 56)	Previous cycle without melatonin (n = 56)	Higher fertilisation rate in melatonin cycle (50% vs 20.2%)*
						No difference in pregnancy rate
Eryilmaz et al. 2011 [137]	Unblinded randomised controlled trial	1^-	60	3 mg melatonin po from day 3–5 until HCG injection (n = 30)	No melatonin (n = 30)	Higher number of oocytes in melatonin group (11.5 vs 6.9)*
						Higher MII oocyte counts (9 vs 4.4)*
						Higher G1 embryo transfer rate (69.3 vs 44.8)*
						No differences in fertilisation, implantation or clinical pregnancy rates
Batioglu et al. 2012 [138]	Single-blinded randomised controlled trial (only embryologists were blinded)	1^-	85	3 mg melatonin po (n = 40)	No melatonin (n = 45)	Higher percentage of MII oocytes in melatonin group (81.9% vs 75.8%)*
						Higher number of G1 embryos (3.2 vs 2.5)*
						No difference in number of oocytes, fertilisation rate or clinical pregnancy rate
Nishihara et al. 2014 [134]	Uncontrolled before - after study	2^-	97	3 mg melatonin po for at least 2 weeks leading up to HCG trigger in second cycle (n = 97)	No melatonin in first cycle (n = 97)	Higher ICSI fertilisation rate in melatonin group (77.5% vs 69.3%)*
						Higher rate of good quality embryos (Day 3) (65.6% vs 48.0%)*
						No difference in maturation rate, blastocyst rate or good quality blastocysts (Day 5)
Combinations with melatonin						
Rizzo et al. 2010 [139]	Unblinded randomised controlled trial	1^-	65	3 mg melatonin daily +2 g myo-inositol po bd +200mcg folic acid po bd from day of GnRH administration (n = 32)	2 g myo-inositol po bd +200mcg folic acid po bd from day of GnRH administration (n = 33)	Higher number of MII oocytes in melatonin group (6.56 vs 5.76)*
						Lower number of immature oocytes (1.31 in vs 1.90)*
						No difference in fertilisation rate, embryos transferred, implantation rate or clinical pregnancy rate
Unfer et al. 2011 [165]	Uncontrolled before - after study	2^-	46	2 g myo-inositol po +200mcg folic acid po in the morning and 3 mg melatonin po +2 g myo-inositol po +200mcg folic acid po in the evening for 3 months leading to second cycle of IVF	No trial medication in first cycle	Higher number of MI and MII oocytes in treatment cycle (3.11 vs 2.35)*
						Higher number of G1 or G2 embryos transferred (0.35 vs 0.13)*
						Clinical pregnancy rate 19.6% in treatment cycle
						No differences in number of oocytes or fertilisation rate
Pacchiarotti et al. 2013 [164]	Double-blinded randomised controlled trial	1^+	388	3 mg melatonin po +4 g myo-inositol po +400mcg folic acid po (n = 178)	4 g myo-inositol +400mcg folic acid po (n = 180)	Higher percentage of mature oocytes in melatonin group (48.2% vs 35.0%)*
						Higher percentage of G1 embryos (45.7% vs 30.4%)*

IVF: In-vitro fertilisation; *NICE*: National Institute for Health and Care Excellence; *statistically significant; *G1*: Grade 1; *G2*: Grade 2; *MI*: Meiosis I; *MII*: Meiosis II; *ICSI*: Intracytoplasmic sperm injection; *HCG*: Human chorionic gonadotrophin; *po*: per oral; *bd*: Twice per day.

trial by using adjuvant combinations as a control group. A prospective non-placebo controlled trial comparing myo-inositol (an insulin sensitizing agent) and folate supplementation to myo-inositol, folate and melatonin found that those in the melatonin group achieved a greater number of mature oocytes, fewer immature oocytes and a greater number of top-quality embryos [139]. While this suggests an independent effect of melatonin, it may be that melatonin acts synergistically with these agents, given that melatonin can enhance the effect of other antioxidants (Figure 1). Indeed, other investigators have shown that myo-inositol may also be a useful treatment for infertility in polycystic ovarian syndrome (PCOS), with improvements observed in the quantity of mature oocytes, the number of top quality embryos and the clinical pregnancy rates [140].

Effects of melatonin on sperm quality

It appears that the reproductive effects of melatonin do not extend only to the female counterpart, with melatonin receptors being demonstrated on spermatozoa [141]. In general, it is accepted that a higher percentage of motile sperm is associated with improved fertilisation rates and Ortiz et al. has shown that the addition of melatonin to seminal samples can improve the overall motility and the percentage of progressively motile spermatozoa [142,143]. Melatonin also appears to inhibit apoptosis in spermatozoa, with a reduction in early apoptotic events being demonstrated in human sperm thus prolonging sperm survival [144]. These effects would serve to improve sperm quality, therefore increasing the probability of successful fertilisation.

Melatonin, through its neutralisation of reactive oxygen and nitrogen species, has been shown in both animal and human studies to improve seminal quality in-vitro. A study investigating the addition of melatonin to semen extender in cryopreserved seminal samples from Holstein bulls resulted in amelioration of the oxidative effects of the freeze-thaw process [145]. Studies in rats also have shown that melatonin has a positive effect on sperm that have been subjected to oxidative stress, improving sperm number, viability and motility [146-148]. Similar results have been found in a small human study in which in-vitro melatonin-treated samples showed a higher percentage of sperm motility and a lower proportion of non-viable spermatozoa [149]. These authors suggested that the mechanism behind their findings was the result of melatonin neutralising reactive nitrogen species [149].

Effects of melatonin on embryo culture media

Following retrieval, the micro-environment that gametes and embryos are cultured in is an essential determinant of subsequent fertilisation and implantation success. Many investigators have studied the impact of melatonin supplementation of in-vitro culture media in porcine, murine and bovine embryo development, overall demonstrating a beneficial effect [150-152].

Bovine studies have found a higher cleavage rate, increased 8-cell embryo yield and an increased number of blastocysts and blastocyst hatching in embryos cultured with melatonin concentrations ranging between 10^{-5} and 10^{-11} M [153-156]. Like supplementation of oocyte culture media, it appears that higher concentrations of melatonin in embryo culture media can be harmful [157].

Therefore, it appears that in-vitro supplementation of embryo culture media with melatonin has a significant impact on the development and quality of embryos, with lower concentrations being more beneficial (and less harmful) than higher ones.

Effects of melatonin on luteal function

Progesterone is an essential hormone in the development of a receptive endometrium and for support of early pregnancy, and without it, pregnancy will fail. In a normal menstrual cycle, this progesterone is provided by the corpus luteum, which develops when the granulosa cells in the ruptured follicle luteinise. A certain level of ROS are required for normal ovulation (follicular rupture) and corpus luteal function. An imbalance of ROS results in oxidative stress and this has been identified as a potential cause of luteal phase defect [158,159].

Studies have also sought to identify the role of melatonin administration during the luteal phase in patients undergoing IVF. A prospective study of 25 women with luteal phase defect compared 14 women who were given 3 mg/d of melatonin from the time of their HCG trigger throughout the luteal phase with 11 women who were given no supplements. The findings showed that melatonin supplementation significantly increased progesterone levels (11.0 ng/ml vs 8.9 ng/ml, $p < 0.05$) [160]. Another study found that melatonin can increase serum progesterone levels in women with a luteal phase defect, but this study did not have a control arm and the observed differences in serum concentrations (<10 ng/ml) were not clinically significant, making the relevance of these findings questionable [161]. Consequently, the application of melatonin for luteal phase support is yet to be confirmed.

Effects of melatonin on pregnancy rates - human studies

Several trials designed to determine the efficacy of melatonin in improving pregnancy rates have considered it in combination with folic acid and myo-inositol, a B complex vitamin synthesized endogenously from glucose [139,162-164].

Rizzo et al. [139] in a prospective trial of 65 women compared myo-inositol and folate supplementation to myo-inositol, folate and melatonin. They found a trend

towards a higher clinical pregnancy rate in the melatonin group, but this did not reach statistical significance.

In a larger double blind randomised controlled trial addressing these agents in PCOS patients undergoing ICSI, Pacchiarotti et al. [164] allocated 178 patients to triple therapy (myo-inositol 4 g, folic acid 400mcg and melatonin 3 mg per day) and 180 patients to myo-inositol and folic acid alone [164]. With this larger sample size, they found higher numbers of mature oocytes (48% vs 35%, $p = 0.008$) and grade 1 embryos (45.7% vs 30.4%, $p = 0.0045$) in patients treated with triple therapy, supporting the role of melatonin in the treatment of infertility caused by PCOS. This does not necessarily demonstrate an independent effect of melatonin on embryo quality or oocyte maturity, and as discussed previously, may represent a synergistic effect with myo-inositol and folic acid, although this has not been proven.

Overall, only a limited number of clinical studies have investigated the use of melatonin to improve pregnancy outcomes in infertile women. These studies have generally been poorly designed, have often compared combination regimens, have investigated a narrow range of melatonin doses and have been unable to conclusively identify an independent positive role for melatonin on clinical pregnancy rates after IVF. There clearly is a need for a large randomised double blind placebo-controlled trial to investigate whether oral melatonin increases clinical pregnancy rates in IVF patients and which dose provides maximal benefit.

Tamura et al. [125] investigated the role of melatonin supplementation in 115 patients who failed to become pregnant in a previous cycle of IVF/ET, with a fertilisation rate of less than or equal to 50%. They used a dose of 3 mg/day in the next IVF cycle from day 5 of the menstrual cycle until oocyte retrieval. The fertilisation rate was significantly higher in the melatonin group when compared with their first cycle ($50.0 \pm 38.0\%$ vs $22.8 \pm 19.0\%$, $p < 0.01$). In addition, intrafollicular melatonin concentrations were significantly increased and the oxidative stress marker 8-OHdG was significantly decreased by melatonin treatment [125]. Furthermore, the pregnancy rate trended towards an improvement in the melatonin group, albeit not reaching statistical significance.

Another prospective longitudinal cohort study addressed the effects on myo-inositol and melatonin supplementation in women who failed to conceive in previous IVF cycles because of poor oocyte quality [165]. Forty six women were treated with myo-inositol 4 g/day and melatonin 3 mg/day for three months and then underwent another IVF cycle. After this treatment, there were statistically significant improvements in the number of mature oocytes and fertilisation rate. The number of top-quality embryos transferred was also higher than the previous cycle. The clinical pregnancy rate after supplementation was 19.6%. Because this was a before-after study and patients were only included if they failed to conceive in their first cycle, it is difficult to comment on the significance of this clinical pregnancy rate as an appropriate control group was not used.

Unfortunately, both studies were of low quality using a before and after comparison with regression to the mean likely explaining observed differences [136].

Systematic reviews and meta-analyses

Only one meta-analysis has been performed specifically assessing the use of melatonin in IVF. This recent systematic review and meta-analysis of five randomise controlled trials found a pooled risk ratio of 1.21 (95% CI 0.98 - 1.50) in favour of melatonin for the outcome of clinical pregnancy rate. However, the authors suggested that the adequacy of the data evaluating the usefulness of melatonin is poor, and that it should not yet be recommended for routine use [166]. While they did not find any worsening of the outcomes of IVF, the authors commented on the lack of live birth rate as an outcome measure as well as the imprecision encountered in all studies considered [166].

On the other hand, melatonin is also known to be remarkably safe, with the Cochrane systematic review and meta-analysis finding no association between antioxidant supplementation and adverse effects for women involved in treatment [88]. This meta-analysis which considered studies of melatonin as well as other antioxidants, found a similar non-statistically significant improvement in clinical pregnancy rate when using any antioxidant (OR 1.30, 95% CI 0.92 - 1.85) with a total sample size of over 2000 patients [88].

Conclusion and future directions

While the beneficial nature of melatonin, an endogenous anti-oxidant, has been known for decades, the investigation into the role of melatonin in the treatment of infertility is still in its infancy. Good quality evidence has emerged from other disciplines indicating the utility of melatonin in the treatment of a variety of medical conditions. For example, a recent phase II double blind placebo controlled randomised trial has shown that melatonin can help reduce chronic pelvic pain in women with endometriosis potentially through its effects on brain-derived neurotrophic factor and beneficial effects on sleep quality [57]. Level II evidence has also determined the effectiveness of melatonin as an analgesic in temporomandibular disorders [167] and as a method of reducing oxidative stress and improving dyspnoea in patients with chronic obstructive pulmonary disease [6]. Despite this, melatonin use in infertility treatment still lacks adequate evidence to recommend routine use.

Infertility treatments are associated with significant levels of reactive oxygen species which have the potential to negatively affect the quality of oocytes and embryos. Melatonin shows promise as an adjunctive therapy in the treatment of infertility. Its unique anti-oxidative characteristics and safety profile make it an ideal potential adjuvant therapy to be further investigated in well designed double blind randomised placebo-controlled trials.

Abbreviations

IVF: In-vitro fertilisation; ART: Assisted reproductive technology; ICSI: Intracytoplasmic sperm injection; ET: Embryo transfer; ROS: Reactive oxygen species; DNA: Deoxyribonucleic acid; GnRH: Gonadotrophin releasing hormone; 8-OHdG: 8-hydroxy- 2′-deoxyguanosine; MI: Meiosis I; MII: Meiosis II; PCOS: Polycystic ovarian syndrome; HCG: Human chorionic gonadotrophin; LH: Luteinising hormone.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SF and LR had significant roles in drafting, revising and authorising this paper for publication. Both authors read and approved the final manuscript.

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Chapter Two

Melatonin in assisted reproductive technologies - Study protocol

2.1 INTRODUCTION

As explained in 1.4, there is a necessity for well-designed placebo-controlled randomised studies to determine if melatonin has a positive impact on human IVF success rates. Such a study should also assess multiple doses of melatonin to ensure that alternative dosing regimens are not superior. To date, no such study has been presented. There are several likely reasons for this. Firstly, such a study, if it were designed to be definitive, would require a large sample size. This could be reduced if a 'higher risk' population (for example, women who respond 'poorly' to IVF treatment) was selected for investigation, as the magnitude of improvement would likely be higher than in a 'low-risk' population. However, randomising patients who have failed multiple expensive cycles of IVF to receive either a placebo or a melatonin dose has previously been considered unlikely to be practical.

Often, patients who are desperate to conceive and who have failed multiple cycles previously, will request treatment with any adjuvant that has the potential to improve their chances of success, particularly if that adjuvant is inexpensive and presumed to be safe. Furthermore, if we were to study patients with multiple failed cycles, this would need to be accounted for in the analysis, which would require a larger sample size.

To overcome this, and to provide a more standardised baseline, I chose to study women undergoing their first cycle of IVF. Because 'higher risk' groups are more difficult to identify in large numbers (and are potentially more difficult to recruit to a randomised and blinded trial), for the purposes of this thesis, I focussed on a relatively 'low risk' group of patients having their first cycle of IVF.

What follows is a detailed description of the trial protocol published prior to commencement of participant recruitment. Detailed information regarding Standard Operating Procedures can be found in *Appendix A* and the Patient Information and Consent Form can be found in *Appendix B*.

2.2 A PILOT DOUBLE-BLIND RANDOMISED PLACEBO-CONTROLLED DOSE-RESPONSE TRIAL ASSESSING THE EFFECTS OF MELATONIN ON INFERTILITY TREATMENT (MIART): STUDY PROTOCOL

Open Access Protocol

BMJ Open

A pilot double-blind randomised placebo-controlled dose–response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol

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ABSTRACT

Introduction: High levels of oxidative stress can have considerable impact on the outcomes of *in vitro* fertilisation (IVF). Recent studies have reported that melatonin, a neurohormone secreted from the pineal gland in response to darkness, has significant antioxidative capabilities which may protect against the oxidative stress of infertility treatment on gametes and embryos. Early studies of oral melatonin (3–4 mg/day) in IVF have suggested favourable outcomes. However, most trials were poorly designed and none have addressed the optimum dose of melatonin. We present a proposal for a pilot double-blind randomised placebo-controlled dose–response trial aimed to determine whether oral melatonin supplementation during ovarian stimulation can improve the outcomes of assisted reproductive technology.

Methods and analyses: We will recruit 160 infertile women into one of four groups: placebo (n=40); melatonin 2 mg twice per day (n=40); melatonin 4 mg twice per day (n=40) and melatonin 8 mg twice per day (n=40). The primary outcome will be clinical pregnancy rate. Secondary clinical outcomes include oocyte number/quality, embryo number/quality and fertilisation rate. We will also measure serum melatonin and the oxidative stress marker, 8-hydroxy-2′-deoxyguanosine at baseline and after treatment and levels of these in follicular fluid at egg pick-up. We will investigate follicular blood flow with Doppler ultrasound, patient sleepiness scores and pregnancy complications, comparing outcomes between groups. This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.

Ethics and dissemination: Ethical approval has been obtained from Monash Health HREC (Ref: 13402B), Monash University HREC (Ref: CF14/523-2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107). Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals.

Trial registration number: ACTRN12613001317785.

Strengths and limitations of this study

- This trial is a well-designed pilot study to achieve biochemically and clinically relevant outcomes.
- Significant preliminary research has been performed in order to appropriately design the study to fill gaps in current knowledge.
- This is the first randomised, placebo-controlled, dose-finding trial of melatonin in the field of infertility worldwide and if successful, has the potential to provide a foundation for future large RCTs.
- A cross-over design will be used as this is known to improve recruitment rates.

INTRODUCTION

Humans are aerobic organisms and when oxygen is utilised in metabolic processes, free oxygen radicals are created as a consequence. These radicals are oxidants and contain 'free' valence electrons, making them highly unstable and therefore reactive, capable of causing injury to cells. This occurs by accepting electrons from another molecule (a reductant) in order to reach stability. In doing so, the chemical structure of the reductant is changed, and may no longer be able to perform its designated function. In fact, often the reductant can then produce or sometimes even become an oxidant having the potential to cause oxidative stress in its own right.[1] Antioxidative agents are present endogenously and can also be administered exogenously. They are designed to reduce free radicals by donating electrons to stabilise the free radical.[2] The term 'reactive oxygen species' (ROS) not only includes free radicals but also molecularly stable non-radical molecules which contain oxygen and

are capable of causing oxidative stress, such as hydrogen peroxide (H_2O_2).[3] While ROS are necessary for essential physiological processes, an overabundance can result in cellular and tissue damage and this is commonly referred to as 'oxidative stress'.[4] Oxidative stress can be measured in bodily fluids by markers including 8–hydroxy-2′-deoxyguanosine (8-OHdg).[5]

Over recent years, the potential role of oxidative stress in the outcomes of assisted reproductive technology (ART) has been gaining increasing attention, in particular with regard to *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). This is perhaps not surprising given that ART exposes both oocytes and embryos to high levels of superoxide-free radicals during gamete and embryo culture.[6] In addition to the culture conditions, *in vitro* the oocytes are not afforded the normal protection of the antioxidant-rich follicular fluid or surrounding cumulus cells, leaving them more susceptible to oxidative damage.[7 8] Reproductive processes such as chromosome segregation (resulting in euploid oocytes), polar body extrusion (necessary for haploid oocytes), fertilisation and early cleavage (to avoid arrested embryos) require energy. As the average age of women seeking fertility treatment rises, the level of ROS and subsequent oxidative stress increases, resulting in mitochondrial DNA mutations which in turn affect ATP production in the oocyte. Without the necessary ATP, required cellular processes cannot occur correctly, having a direct effect on the quality of the oocyte, embryo and the final outcome of the IVF procedure.[9 10]

It has been shown that oocyte quality begins to deteriorate immediately following ovulation. This has been thought to be an inflammatory-driven oxidative stress process[11] whereby the production of cytokines and proteases is associated with an increase in ROS which in turn impair oocyte maturation.[12–14] Evidence of oxidative damage in *in vitro* oocytes has been shown to exist as early as 8 h after ovulation.[15] Nor surprisingly it has been shown that high levels of the oxidative stress in the follicular fluid of infertile women are associated with poor oocyte maturation and embryo quality, and that inducers of oxidative stress can be used to inhibit oocyte maturation.[5 12] Together, these observations highlight the importance of oxidative stress in the developing follicle and in the future success of fertilisation, whether *in vivo* or *in vitro*.

Recently, it has been shown that melatonin, a neurohormone secreted from the pineal gland, has important oxygen-scavenging properties which naturally mitigate oxidative stress by both neutralising ROS in human tissues and by inducing endogenous antioxidant enzymes.[16–18] Melatonin has been shown to be beneficial as an adjuvant therapy in the management of many medical conditions in which oxidative stress has been implicated, including diabetes, glaucoma, irritable bowel syndrome and fertility.[19–22]

The effects of melatonin supplementation on culture media,[12 21 23–27] gametes,[5 15 28] embryos[15 29 30] and luteal function[31–35] have been identified as useful areas for investigation. Human clinical studies have begun recently, with emphasis on oral supplementation of melatonin during the stimulation cycle and its effects on oocyte and embryo quality.[12 36–39]

Importantly, melatonin has a remarkably benign safety profile in animal and human studies. A mouse study investigating dose-related effects of melatonin on ovarian grafts found that doses at 200 mg/kg/day have immunosuppressant effects, leading to prolongation of graft survival, however, also resulted in a reduction in healthy follicles. They concluded that doses of greater than 20 mg/kg/day resulted in a reduction in healthy follicles and ovarian size.[40] The maternal lowest observed adverse effect level, the lowest level at which an adverse event was observed, appears to be around 200 mg/kg/day, equivalent to over 1 g/day for humans. Furthermore, in rodent studies, no significant adverse effects on either the embryo or fetus have been reported for up to doses of 200 mg/kg/day.[41] There have been several randomised controlled trials (RCTs) addressing high doses of melatonin in human adults and children,[42–46] and reports have established that toxicity levels in humans are not reached until a daily dose of at least 5 mg/kg.[47]

RATIONALE

In the USA, in 2011, only 1 in 4 of all IVF cycles resulted in clinical pregnancy with less than 1 in 5 cycles resulting in live birth.[48] That is, 4 out of 5 IVF cycles do not result in a live baby. Clearly there is much room for further improvement, if only to meet societal expectations and reduce healthcare costs. Continued research into novel adjuvant therapies designed to improve IVF outcomes is therefore merited. Several human and animal studies support the use of melatonin in the treatment of infertility, with beneficial actions being largely attributed to its antioxidant properties. Furthermore, melatonin receptors are found on granulosa cells, oocytes and embryos,[49] with intrafollicular melatonin concentrations being significantly higher than those found in serum, again suggesting a physiological role in reproduction.[39 50 51] The human studies addressing the use of melatonin in infertility treatment undertaken to date have been small and not placebo-controlled. None have attempted to identify an optimal dose.[36–38 52] Furthermore, interpretation of any outcomes has been hampered by the within-patient comparison design, where patients act as their own controls.[12 39 53]

We have designed this study to be the first randomised, placebo-controlled, dose-finding clinical trial to investigate the effect(s), if any, of oral melatonin on the clinical pregnancy rate following IVF/ICSI treatment.

AIMS

The overall aim of this trial is to determine whether oral melatonin administration can improve the outcomes of

IVF/ICSI. In order to address this, we will determine whether melatonin administration has a dose–response effect in women undergoing IVF/ICSI on:

1. Biochemical markers of follicle and oocyte health (melatonin, 8-OHdg, progesterone, oestradiol);
2. Sonographic markers of follicle health;
3. Patient sleepiness;
4. Pregnancy rate following IVF/ICSI.

METHODS AND ANALYSES

This protocol has been designed in accordance with the SPIRIT 2013 Guidelines.[54] Recruitment will start in July 2014. This will occur over 2 years. Analysis and dissemination will occur after this period of time. The study is expected to be completed by February 2017.

STUDY DESIGN

Pilot phase II cross-over double-blinded randomised placebo-controlled dose–response trial.

PARTICIPANTS

We plan to recruit a total of 160 infertile women undergoing IVF/ICSI.

STUDY SETTING

Patients will be recruited from Monash IVF infertility clinics in Melbourne, Australia over a period of 2 years.

INTERVENTIONS TO BE MEASURED

This trial will have four arms. All capsules will be indistinguishable from each other.

1. Placebo capsule taken twice per day;
2. 2 mg melatonin capsule twice per day (4 mg/day total);
3. 4 mg melatonin capsule twice per day (8 mg/day total);
4. 8 mg melatonin capsule twice per day (16 mg/day total).

PRIMARY OUTCOME

Clinical pregnancy rate,[55] defined as the presence of a live pregnancy in the uterine cavity at a transvaginal ultrasound at 6–8 weeks' gestation.

SECONDARY OUTCOMES

See table 1 for details.

Table 1 Clinical secondary outcomes

Clinical outcomes	Definition
Live birth rate	Birth of a live baby after 24 weeks gestation
Miscarriage rate	Loss of a diagnosed clinical pregnancy before 20 weeks gestation
Sleepiness score	Based on Karolinska[56] sleepiness scale during trial medication administration
Pregnancy complication and adverse events rates	Including OHSS, multiple pregnancy, congenital or chromosomal abnormalities, stillbirth, pre-eclampsia, delivery before 34 weeks, delivery between 34 and 37 weeks, placenta praevia, gestational diabetes, low birth weight
Embryological outcomes	
Total number of oocytes collected	
Oocyte maturity	
Total number of embryos	
Embryo quality	
Fertilisation rate	The proportion of oocytes that become fertilised
Utilisation rate	Proportion of zygotes undergoing embryo transfer or cryopreservation to oocytes fertilised
Biochemical outcomes	
Biochemical pregnancy rate	Presence of serum hCG level of >25 IU/L on day 16 after embryo transfer
Melatonin levels in serum	At baseline and prior to oocyte collection
Melatonin levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
8-OHdg levels in serum	At baseline and prior to oocyte collection
8-OHdg levels in follicular fluid	Taken from leading follicle from each ovary at time of oocyte collection
Oestradiol and progesterone levels in serum	Taken at baseline, during treatment and at the time of oocyte collection
Sonographic outcomes	
Follicular blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection
Uterine artery blood flow	Measured on last transvaginal ultrasound with power Doppler prior to oocyte collection

hCG, human chorionic gonadotropin; OHSS, ovarian hyperstimulation syndrome; 8-OHdg, 8-hydroxy-2′-deoxyguanosine.

SAMPLE SIZE

As this is a pilot dose-finding study, without precedence on which to base accurate power calculations, a power calculation has not been performed. Part of our aim is to provide clarification allowing for larger randomised trials to be designed in the future. We hypothesise that melatonin will have a positive dose–response effect on clinical pregnancy rates after IVF/ICSI. We have chosen a convenience sample of 160 participants, 40 in each of the four groups, in order to assess plausible morphological, biochemical and sonographic surrogate markers for oocyte quality, as well as to provide an indication of the effect size for each dose for our primary outcome, clinical pregnancy rate. Conservatively estimating that, in total, 50% of patients (n=80) do not get pregnant after their first cycle, we may assume that 60 patients will participate in the cross-over arm (allowing for drop-outs) in their second cycle and be allocated to a different treatment group.

INCLUSION CRITERIA

1. Undergoing first cycle of IVF or ICSI;
2. Age between 18 and 45;
3. Body mass index between 18 and 35;
4. Undergoing a gonadotrophin releasing hormone (GnRH) antagonist cycle (without oral contraceptive pill (OCP) scheduling).

EXCLUSION CRITERIA

1. Current untreated pelvic pathology—moderate-to-severe endometriosis, submucosal uterine fibroids/polyps assessed by the treating specialist to affect fertility, pelvic inflammatory disease,

	Study Period						
	Enrolment	Trial medication begins	Post allocation				Close out
Timepoint (days)	-7	0	8-10	14	19	68	299
ENROLMENT							
Eligibility Screen	x						
Informed Consent	x						
Allocation	x						
INTERVENTIONS							
Trial Medication[a]		x	———	x			
ASSESSMENTS							
Baseline blood tests	x						
Blood tests after treatment				x			
Follicular fluid melatonin and 8-OHdg				x			
Measurement of follicular and uterine blood flow			x				
Oocyte assessments				x			
Embryo assessments				x	———	x	
Sleepiness scores		x	———	x			
Pregnancy complication rates and outcomes						x	——— x
Followup hCG					x	———	x

Figure 1 Participant timeline schematic—first cycle. [a]Placebo, melatonin 2 mg twice daily, melatonin 4 mg twice daily, melatonin 8 mg twice daily. Note: patients who are not pregnant after their first cycle will be offered a second cycle with a different trial medication (figure 2). hCG, human chorionic gonadotropin; 8-OHdg, 8-hydroxy-2′-deoxyguanosine.

uterine malformations, Asherman's syndrome and hydrosalpinx.
2. Currently enrolled in another interventional clinical trial.
3. Concurrent use of other adjuvant therapies (e.g. Chinese herbs, acupuncture).
4. Current pregnancy.
5. Malignancy or other contraindication to IVF.
6. Autoimmune disorders.
7. Undergoing preimplantation genetic diagnosis.
8. Hypersensitivity to melatonin or its metabolites.
9. Concurrent use of any of the following medications:
   A. Fluvoxamine (e.g. luvox, movox, voxam);
   B. Cimetidine (e.g. magicul, tagamet);
   C. Quinolones and other CYP1A2 inhibitors (ciprofloxacin, avalox);
   D. Carbamazepine (e.g. tegretol), rifampicin (e.g. rifadin) and other CYP1A2 inducers;
   E. Zolpidem (e.g. stilnox), zopiclone (e.g. imovane) and other non-benzodiazepine hypnotics.
10. Inability to comply with trial protocol.

CONCOMITANT CARE

Aside from trial protocol and inclusion/exclusion criteria listed above, standard concomitant care, such as folic acid supplements, is permitted.

PARTICIPANT ENROLMENT

This trial will begin recruitment at the level of the infertility specialist. Once identified as requiring IVF/ICSI, and satisfying inclusion and exclusion criteria, the patient will be provided with detailed written information about the trial protocol. After approximately 1 week, the patient will be approached and written informed consent will be obtained by the principle investigator at the first visit with the infertility clinic nurse. Baseline blood tests will be taken and basic demographic information including aetiology of infertility will be recorded. The patient will then be randomised by the principle investigator (see below) to one of the study arms and provided with the trial medication. The participant will start taking the trial medication on the day that ovarian stimulation injections begin (days 2–3 of menses) at 08:00 and 22:00 each day, with the last capsule being taken at 22:00 the night before oocyte collection.

For a summarised participant timeline, see figures 1 and 2.

ALLOCATION CONCEALMENT, BLINDING AND RANDOMISATION

Association of particular treatment effects (e.g. sleepiness) with certain randomisation codes may become apparent during the trial, and to prevent further allocation bias, proper concealment of treatment allocation is necessary. Each treatment arm will be randomly allocated a letter (A, B, C or D) by way of opaque sealed envelope. Randomisation will be computerised and patients will be randomised at a ratio of 1:1:1:1 to one of the groups, A–D, using the minimisation method, a

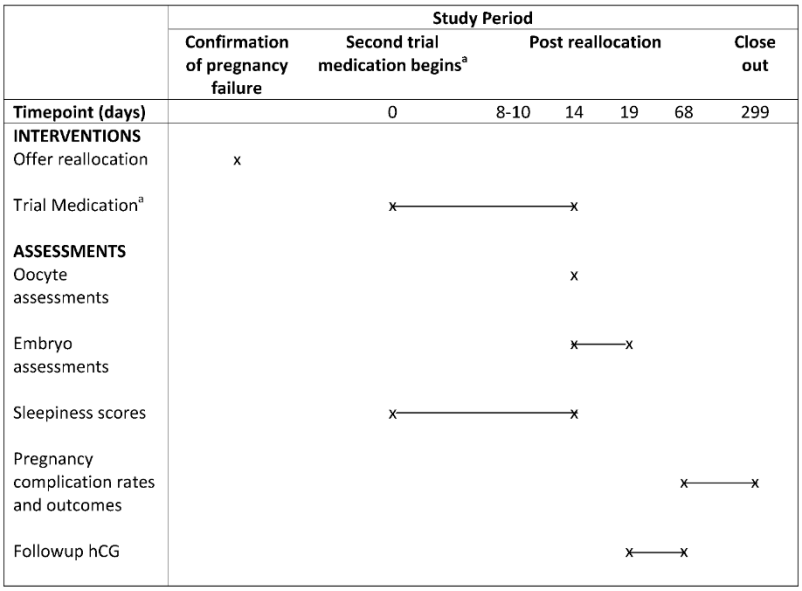

Figure 2 Participant timeline schematic—second cycle in those not pregnant after their first cycle. [a]For participants who do not become pregnant in their first cycle, each will be offered allocation to the next treatment arm for their second cycle (i.e. A will be allocated to B, B to C, C to D and D to A). ET, embryo transfer; hCG, human chorionic gonadotropin.

method of randomisation accounting for factors known to affect the outcome used in small trials to prevent selection bias.[57]

With a cross-over design, improved patient recruitment is very likely because all non-pregnant participants will be guaranteed at least one cycle with one of the doses of melatonin.[58 59] After completing the first study cycle, those participants who are not pregnant will be recruited for the cross-over cycle in which they will be assigned the next treatment arm. For example, if a participant were allocated to treatment group A in their first cycle and did not become pregnant, they would be offered allocation to group B in their second cycle and so on. The half life of melatonin is short, being completely eliminated from the body shortly after 24 h.[60] Once patients have had their first stimulated cycle of treatment, a second stimulated cycle will routinely not start until at least 4 weeks after the negative pregnancy test (approximately 6 weeks after last melatonin dose), much longer than the elimination time for melatonin. This will be a sufficient washout period prior to inclusion in a second cycle.

Participants will be blinded by receiving identical-appearing unmarked capsules. All trial researchers will be blinded to treatment allocation group until after analyses are performed at the completion of the trial. A Data Safety Monitoring Committee (see *Adverse events and data safety and monitoring* section for details) and dispensing pharmacy will be aware of allocation to allow identification of any unbalanced or multiple serious adverse events that may necessitate emergency unblinding of researchers and participants.

ADHERENCE AND RETENTION

In order to ensure the integrity of study data, participant adherence to trial protocol will be assessed on a medication administration record updated daily by the participant. At oocyte collection, participants will return medication bottles and compliance will be confirmed by counting remaining tablets. This will be recorded on individual patient compliance forms and patient record forms. Every reasonable effort will be made from the time of enrolment until the end of follow-up to maintain contact with and maintain participant's participation in the trial.

ANALYSIS PLAN

SPSS V.22.0 (IBM, Armonk, New York, USA) will be used for data analysis. Primary analysis will occur by the intention-to-treat principle. A secondary analysis will be performed for 'actual treatment received'. We also intend to perform a separate subanalysis on the data from the first cycle only. Statistical analyses will be performed using χ^2 tests for dichotomous categorical outcome variables. Clinical and demographic data will be analysed with parametric tests if they are normally distributed (analysis of variance (ANOVA) with Bonferroni adjustment), otherwise appropriate non-parametric tests will be implemented (Kruskal-Wallis for between group analyses). Within patient changes (paired data), e.g. when comparing serum levels of melatonin at baseline and after treatment, will be compared using repeated measures ANOVA or Wilcoxon signed-rank test depending on normality of data. A $p<0.05$ will be considered statistically significant. Where statistically sound, adjusted ORs will be calculated to account for confounders or effect modifiers. To account for missing data, two analyses will be run, with the first excluding missing values and the second by imputation of missing values to determine how conclusions are affected. The SPSS multiple imputation routine (MCMC algorithm known as Fully Conditional Specification) will be used to handle missing data.

ADVERSE EVENTS AND DATA SAFETY AND MONITORING

An independent Data Safety and Monitoring Committee (DSMC) has been established at Monash Health in order to monitor occurrences of adverse events. The principle investigator will be available by telephone at all times during the trial and participants will be provided with contact details in case of any adverse events. Participants will also be interviewed at the time of their ultrasound and the day of oocyte collection to ascertain the occurrence of any adverse events. Serious adverse events will be recorded separately and followed up until resolution. Such events will be reported to the Monash Health, Monash University, Monash Surgical Private Hospital and Epworth HealthCare Human Research Ethics Committees, the Monash Health DSMC, the Monash Health Therapeutics Committee and, if directed by HREC, to the Australian Therapeutics and Goods Administration.

TRIAL MODIFICATION AND DISCONTINUATION

In the absence of adverse events, the medication regimen will not be modified once started. If other protocol changes are deemed necessary by the investigating team, ethics approval will be sought from all approving Human Research Ethics Committees. Once approved, protocol amendments will be notified to all investigators, administrators (e.g. dispensing pharmacy) and trial participants via telephone.

Patients are permitted to discontinue their inclusion in the trial at any point in time at their request or following an unexpected serious adverse event as described above. The DSMC will perform an interim analysis specifically assessing adverse events after 50% of participants have been recruited. Otherwise, the trial will cease follow-up once patients have given birth from a trial treatment cycle.

DATA COLLECTION, INFORMED CONSENT FORMS AND CONFIDENTIALITY

Data will be recorded in hardcopy and electronic form. Hardcopies will be stored in a secured filing cabinet at the administering institution. Electronic copies will be stored in encrypted files on a password protected computer. All data will be kept for 15 years; following this time, hardcopies will be destroyed by shredding or burning and electronic copies will be deleted by formatting. Organic samples will be stored until analysis in a secured locked temperature-controlled freezer at the Monash Institute of Medical Research that can only be accessed by authorised staff. Samples and participant records will not contain any directly identifiable information. No additional biological samples will be kept for use in ancillary studies.

For access to data collection forms, including sleep diaries, medication compliance and informed consent forms, please contact the principle investigator.

ETHICS AND DISSEMINATION

Data analysis, interpretation and conclusions will be presented at national and international conferences and published in peer-reviewed journals. De-identified summary results will also be made publically available on the Monash IVF website.

DISCUSSION

The Melatonin in ART (MIART) trial has the potential to improve IVF treatment protocols, with both immediate and translational benefit to patients. If melatonin is found to improve outcomes after IVF/ICSI, it may become a routine part of management of the infertile couple. We aim to set precedence and a framework by which others may structure further investigation into melatonin and other adjuvant therapies in the future. The intention of this study is as a pilot trial, primarily designed to provide unbiased data as a foundation for further research into this area. It is anticipated that the effect size observed in this trial will be useful to more appropriately power subsequent RCTs with the most effective dose of melatonin. In summary, MIART will be the first trial designed to determine a dose–response relationship of melatonin on biochemical and physiological markers of follicle health and also clinical pregnancy rates.

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Contributors The idea for the trial was conceived by SF, LR and EW. SF was involved in research design and primary writing of study protocol and manuscript. TO was involved in the design and writing of the manuscript. LR and EW were involved in the trial design, writing, editing and approval of the final manuscript.

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Competing interests SF is supported by an NHMRC Postgraduate Scholarship. BV and LR are minority shareholders in Monash IVF. LR has received unconditional research and educational grants from MSD and Merck Serono.

Ethics approval Ethical approval has been obtained from Monash Health HREC (Ref:13402B), Monash University HREC (Ref: CF14/523-2014000181) and Monash Surgical Private Hospital HREC (Ref: 14107).

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Chapter Three

Clinical pregnancy and clinical outcomes

3.1 THE EFFECT OF MELATONIN ON CLINICAL PREGNANCY RATE

As discussed in 2.2, melatonin is commonly used in IVF treatment because of a perceived benefit, by patients and clinicians, on IVF success rates. Hence, the primary outcome of the MIART Trial was clinical pregnancy rate, defined as the presence of a live intrauterine pregnancy identified on a first trimester ultrasound scan after 7 weeks' gestation. I was also interested in adverse event rate, as the safety of high doses of melatonin have been poorly documented in the IVF population, and if melatonin were to be a successful treatment, its use would need to be proven to be safe before entering routine clinical practice. The effect on oocyte and embryo number and quality is also reported.

To add plausibility and credibility to this hypothesis by proving medication penetration into target tissues, I also determined absolute changes in serum melatonin, oestradiol (E2), progesterone (P4) and follicular fluid concentrations of these hormones. A recently published study has suggested that melatonin concentrations in follicular fluid on the day of oocyte retrieval correlated positively with oocyte and embryo parameters, but this did not result in an increase in the percentage of high quality embryos (Tong, et al., 2017).

In order to truly blind participants and investigators, all active trial medications were compounded to appear the same by Orrong Compounding Pharmacy, Melbourne, Australia. The melatonin content of all formulations was independently verified (Australian Life Sciences, NSW, Australia) (Appendix E). Melatonin is approved by the Therapeutic Goods Administration (TGA) as a prescription-only (Schedule 4) medication. However, as a specifically formulated version was used for this trial under a Clinical Trial Notification (CTN) authority, it does not carry TGA approval for administration outside this context.

3.2. CROSSOVER

In order to encourage patient recruitment, a 'crossover' arm was included in the trial protocol. If a patient was not successful in achieving a pregnancy in their first cycle, they were offered

inclusion into this arm where they would be guaranteed a different dose of trial medication. In this way, all patients had the option to receive at least one dose of melatonin over two cycles. The second dose was allocated blindly and in sequence. It was expected that several women may wish to participate in the crossover arm of the study, however, only four women (2.7%) did. None of these women became pregnant in their second cycle. Because of the very limited uptake of this option, these results were not considered significant enough to publish or to add to the main analysis.

This low uptake in the crossover arm did not appear to be due to adverse events. Rather, it seems that women were reluctant to participate in the trial for a second cycle after failing one cycle. In addition, some participants became ineligible in their subsequent cycle as their specialists commenced them on other adjuvant therapies (including melatonin) in these cycles.

3.3 MELATONIN IN ASSISTED REPRODUCTIVE TECHNOLOGY: A PILOT DOUBLE-BLIND RANDOMISED PLACEBO-CONTROLLED CLINICAL TRIAL

Journal of Pineal Research

Melatonin in Assisted reproductive technology: A pilot double-blind randomised placebo-controlled clinical trial

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Keywords:	melatonin, assisted reproductive technology, in-vitro fertilisation, clinical pregnancy, follicular fluid

Abstract

Because of its potent oxygen-scavenging abilities, we sought to determine whether oral melatonin can increase clinical pregnancy rate (CPR) after IVF and what dose might be most effective. This was a double-blind, dose-finding, placebo-controlled randomised clinical trial enrolling 160 women having their first cycle of IVF or intra-cytoplasmic sperm injection (ICSI). Women were randomised to receive placebo (n=40), melatonin 2mg (n=41), melatonin 4mg (n=39) or melatonin 8mg (n=40) orally twice per day (BD) during ovarian stimulation. Primary outcome was CPR. Secondary outcomes included serum and follicular fluid (FF) melatonin concentrations, oocyte/embryo quantity/quality and live birth rate (LBR). Compared with placebo, mean FF melatonin concentration in the highest dose group (8mg BD) was 9-fold higher ($P < 0.001$). Despite this, there were no differences between the groups in total oocyte number, number of MII oocytes, number of fertilised oocytes, or the number or quality of embryos between the groups. There was no difference in CPR or LBR between any of the four groups (p=0.5). When taking any dose of melatonin, the CPR was not statistically significantly different compared with placebo (21.7% vs 15.0%, OR 1.57 (95% CI 0.59, 4.14), p=0.4). Therefore, despite a 9-fold increase in FF melatonin concentration, there were no statistically significant differences in clinical outcomes. Because this was a small study, a beneficial effect of melatonin on IVF pregnancy rates cannot yet be excluded. Therefore, we suggest that the use of melatonin in this setting should be restricted to larger clinical trials.

Introduction

Despite significant advances in in vitro fertilisation (IVF), only about a quarter of all IVF cycles result in a clinical pregnancy and less than one in five cycles in live birth.[1] Recently, interest has grown in the effects of oxidative stress on the success rates of IVF. IVF exposes oocytes and embryos to high concentrations of reactive oxygen species (ROS) during gamete and embryo culture.[2] Oxidative stress in the follicular fluid (FF) of infertile women are associated with poor oocyte maturation and embryo quality, and inducers of oxidative stress inhibit oocyte maturation.[3, 4] It has been suggested that anti-oxidant therapy might lessen detrimental effects of excessive ROS and so improve success rates.[5]

Melatonin can mitigate oxidative stress by neutralising ROS in animal[6-8] and human tissues by inducing endogenous anti-oxidant enzymes.[9] Melatonin has been suggested as an adjuvant therapy in the management of diverse medical conditions in which oxidative stress has been implicated, including neural tube defects, diabetes, glaucoma, irritable bowel syndrome, and infertility.[10-14] That melatonin receptors are found on granulosa cells, oocytes and embryos,[15] suggests that it may have a physiological role in reproduction. What that role is, if any, and whether it relates to antioxidant protection remains unknown. Nonetheless, it is intriguing that higher concentrations of melatonin in the ovarian follicle are associated with higher follicular progesterone and lower oestradiol concentrations.[16]

Several human and animal studies support the use of melatonin in the management of infertility.[4, 5, 17-20] Beneficial effects, if any, have been largely attributed to its oxygen scavenging properties.[9] However, clinical trials addressing the use of melatonin in IVF have been small, lack blinding and were not placebo-controlled.[4, 5, 17, 18] In addition, interpretation of some trial outcomes has been hampered by poor study design, where participants have acted as their own controls.[4, 18, 21] Importantly, there have also been no attempts to define an optimal dosing regimen,[22] and considering the short half-life of melatonin,[23] a single daily dose may not provide sustained protection from oxidative stress.

The lack of reliable treatment effect sizes prompted us to undertake a pilot study[24] with a randomised, double-blinded, placebo-controlled dose-finding trial design to (a) estimate the sample size and optimal melatonin dose needed for a future trial, (b) examine the plausibility of the effectiveness of the intervention through the measurement of secondary outcomes, including follicular fluid concentrations of melatonin and quantity/quality of oocytes and embryos, (c) assess the recruitment rate, trial adherence and retention, and (d) record adverse maternal and fetal outcomes of twice daily high dose melatonin administration.

Materials and Methods

Participants

Women were recruited at their first clinical contact visit. Baseline demographic data is detailed in Table 3.1.

TABLE 3.1: DEMOGRAPHICS

Treatment arm	Placebo BD N=40	Melatonin 2mg BD N=41	Melatonin 4mg BD N=39	Melatonin 8mg BD N=40	Total N=160
Age, Mean (SD)	35.2 (4.2)	35.0 (4.1)	36.0 (4.2)	35.4 (4.4)	35.4 (4.2)
BMI, Mean (SD)	24.5 (4.8)	24.6 (4.0)	24.6 (4.5)	24.6 (3.9)	24.6 (4.3)
Gravidity 0	22 (55.0)	24 (58.5)	26 (66.7)	24 (60.0)	96 (60.0)
Gravidity ≥1	18 (45.0)	17 (41.5)	13 (33.3)	16 (40.0)	64 (40.0)
Parity 0	32 (80.0)	35 (85.4)	33 (84.6)	32 (80.0)	132 (82.5)
Parity ≥1	8 (20.0)	6 (14.6)	6 (15.4)	8 (20.0)	28 (17.5)
Current smoker N (%)	4 (10.0)	2 (4.9)	1 (2.6)	2 (5.0)	9 (5.6)
Night shift worker N (%)	1 (2.5)	1 (2.4)	2 (5.1)	3 (7.5)	7 (4.4)
Type					
IVF N (%)	16 (40.0)	12 (29.3)	18 (46.2)	13 (32.5)	59 (36.9)
ICSI N (%)	24 (60.0)	25 (61.0)	21 (53.8)	26 (65.0)	96 (60.0)
Both IVF and ICSI	0 (0.0)	4 (9.8)	0 (0.0)	1 (2.5)	5 (3.1)
Ethnicity					
Caucasian	20 (50.0)	30 (73.2)	28 (71.8)	28 (70.0)	106 (66.3)
Asian and South-Asian	14 (35.0)	9 (22.0)	9 (23.1)	7 (17.5)	39 (24.4)
Other	6 (15.0)	2 (4.9)	2 (5.1)	5 (12.5)	15 (9.4)
Aetiology					
Endometriosis	6 (15.0)	7 (17.1)	5 (12.8)	5 (12.5)	23 (14.4)
PCOS	1 (2.5)	4 (9.8)	1 (2.6)	4 (10.0)	10 (6.3)
Tubal	7 (17.5)	8 (19.5)	4 (10.3)	5 (12.5)	24 (15.0)
Male factor	7 (17.5)	13 (31.7)	10 (25.6)	14 (35.0)	44 (27.5)
Ovulatory	1 (2.5)	1 (2.4)	0 (0.0)	1 (2.5)	3 (1.9)
Social[a]	1 (2.5)	2 (4.9)	4 (10.3)	2 (5.0)	9 (5.6)
Idiopathic	19 (47.5)	17 (41.5)	20 (51.3)	14 (35.0)	70 (43.8)

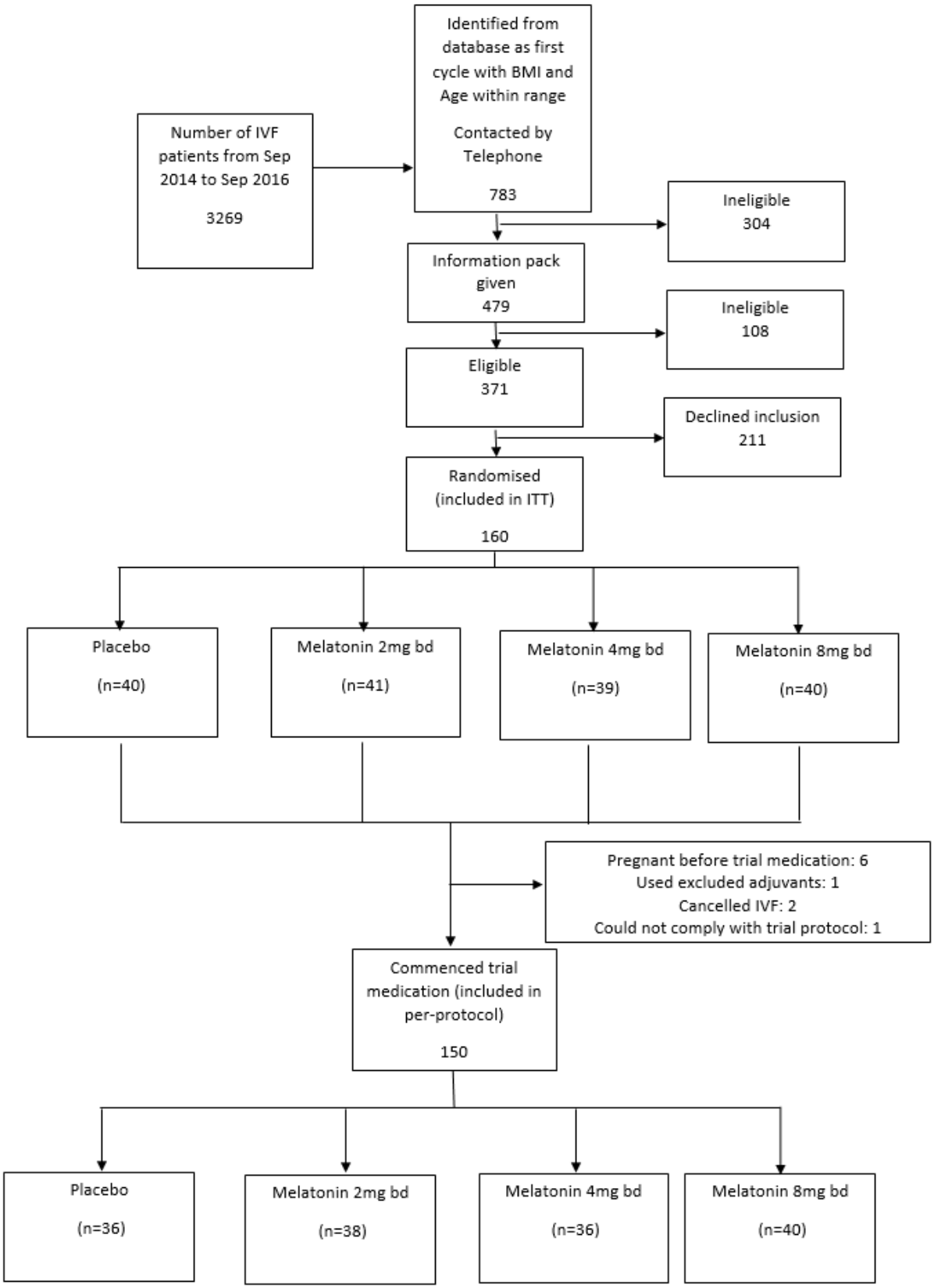
[a] Includes same-sex couples and single women; IVF: in-vitro fertilisation; ICSI: intracytoplasmic sperm injection; BD: twice per day; PCOS: polycystic ovarian syndrome

Women were eligible if they (i) were undergoing their first cycle of IVF/ICSI, (ii) were undergoing an antagonist cycle, (iii) were aged between 18 and 45, and (iv) had a BMI between 18 and 35. Exclusion criteria were: untreated endometriosis, uterine malformations, large distorting fibroids or endometrial polyps, autoimmune disease, concurrent use of other adjuvant therapies, malignancy, preimplantation genetic screening, known sensitivity to melatonin, or if concurrently taking medications known to interact with melatonin (antidepressants, antiepileptics or hypnotics).[24]

Recruitment

We planned to recruit 160 women, 40 in each group. 3269 women underwent their first cycle of IVF/ICSI at Monash IVF between September 2014 and September 2016. Following several steps of eligibility assessment, 371 were found to be eligible, of which 211 declined to participate, leaving 160 to be randomised for intention-to-treat (ITT) analysis (Figure 3.1). Ten (6.3%) of these women were withdrawn after randomisation but before commencement of trial medication because they subsequently met exclusion criteria (pregnant before trial medication, n=6; used excluded adjuvants, n=1; cancelled IVF, n=2; could not comply with trial protocol, n=1), leaving a total of 150 women for per-protocol (PP) analysis (Figure 3.1).

FIGURE 3.1: RECRUITMENT FLOWCHART



ITT: intention-to-treat; PP: per-protocol; BD: twice daily; IVF: in-vitro fertilisation

Blinding and Randomisation

Each dose of trial medication was randomly designated a letter ('A' to 'D') using a random number generator by an independent Data Safety and Monitoring Board (DSMB). This allocation was only known to the DSMB and the hospital pharmacy responsible for labelling and dispensing the medication until after completion of the trial. All staff and participants remained blinded throughout the trial. All medication bottles and capsules were of identical appearance.

To prevent selection bias, randomisation was performed using the minimisation method.[25] Weighted minimisation was performed using age (weighting of 20), parity (weighting of 10), BMI (weighting of 5) and smoking status (weighting of 1) in real-time using minimisation software (MUI Online Minimisation®, powered by Qminim®).

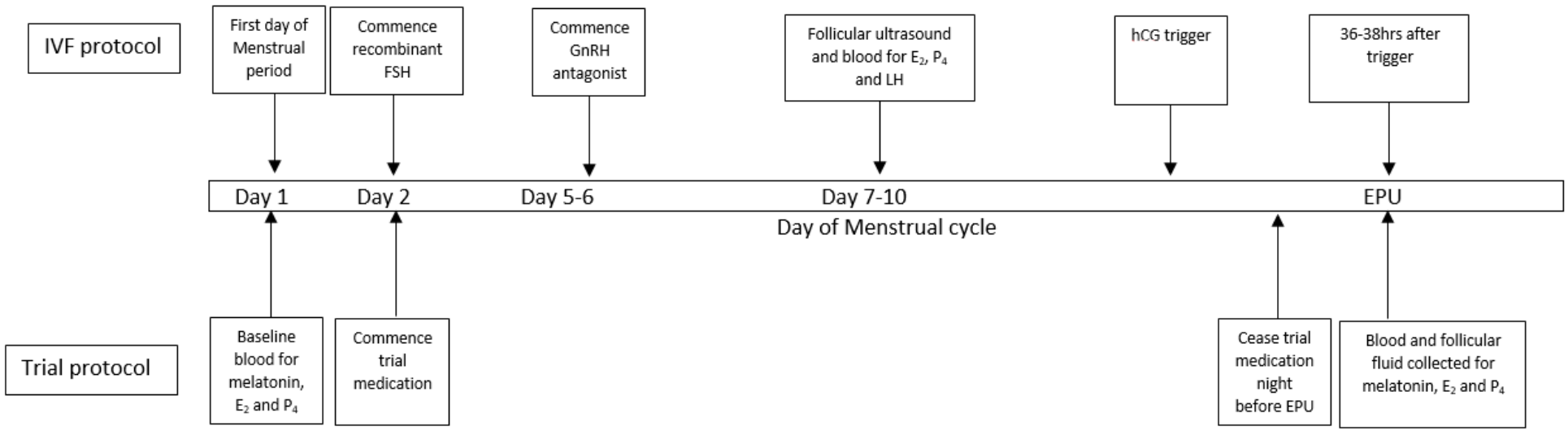
Outcome measures

The primary outcome was CPR (presence of a live intrauterine pregnancy detected on transvaginal ultrasound scan at 6-8 weeks' gestation). The secondary outcomes were LBR, biochemical pregnancy rate (BPR; serum HCG≥25IU/ml >9 days after ET), oocyte and embryo number and quality, number of oocytes fertilised, number of embryos utilised, pregnancy complications, and adverse events including cycle cancellations.[24]

Administration of trial medication and compliance

All active formulations of trial medication were 'sustained release' (Orrong Compounding Pharmacy, Melbourne, Australia). The melatonin content of all formulations was independently verified (Australian Life Sciences, NSW, Australia). Placebo capsules were composed of methylcellulose. Each participant was instructed to take one capsule twice per day (once between 08:00 and 10:00 and once between 20:00 and 22:00) from day two of their stimulation cycle until the night before oocyte retrieval (Figure 3.2). Each participant kept a diary documenting compliance and adverse events.

FIGURE 3.2: IVF AND GENERAL TRIAL PROTOCOL



IVF: in-vitro fertilisation; FSH: follicle stimulating hormone; GnRH: gonadotrophin releasing hormone; hCG: human chorionic gonadotrophin; E_2: oestradiol; P_4: progesterone; EPU: oocyte retrieval

Ovarian stimulation and oocyte retrieval (EPU) protocol

All patients received GnRH antagonist cycles with recombinant FSH and a HCG trigger administered 36 hours before EPU (Figure 3.2). EPU was cancelled if there were fewer than three follicles >17mm or if there was risk of severe OHSS. Transvaginal ultrasound-guided EPU was performed under general anaesthetic.

Collection of blood

Blood was collected to assess melatonin, oestradiol (E2) and progesterone (P4) concentrations on the day of recruitment, day 8-9 of ovarian stimulation and on the day of EPU (prior to general anaesthetic). Blood was transported in light-shielded containers, centrifuged at 1800g at 21°C for 15 minutes and frozen in aliquots at -80°C.

Collection of FF

At EPU, FF was collected from the single largest, most accessible follicle. The volume of collection media (Sydney IVF Follicle Flush Buffer, Cook Medical Australia) was standardised to allow for accurate concentration comparisons between samples. Following EPU, samples were immediately transported on ice in shielded containers, centrifuged at 400g at 4°C for 7 minutes to remove cellular debris and aliquoted at -80°C.

Assays

Serum melatonin concentrations were determined by radio-immunoassay (Buhlmann®, RK-MEL2, Switzerland), according to manufacturer's instructions. The extraction efficiency of the assay was >90%, with an estimated functional sensitivity (CV=10%) of 0.9pg/ml and an estimated analytical sensitivity of 0.3pg/ml.

Serum steroid concentrations were assayed with Chemiluminescent Microparticle Immunoassays (Architect iSystem, Illinois, USA) (progesterone sensitivity <0.1ng/ml; oestradiol sensitivity <10pg/ml).

Oestradiol concentration in FF was determined using mass spectrometry (AbSciex Triple Quad 5500 LC/MS/MS system) following solvent extraction. Progesterone was determined using a Beckman Coulter® competitive binding immunoenzymatic assay performed on a Unicel DXI® 800 analyzer (Lane Cove, Australia).

Embryology procedures and assessments

Standard IVF or ICSI was performed using routine protocols. Experienced embryologists scored oocyte maturity and developing embryos for morphology on Day 3 and Day 5. Embryo quality was graded by blinded embryologists from 'A'-'D'. 'X' was used to describe zygotes that arrested before day 3. Good quality embryos were defined as those scored 'A' or 'B'. Embryo transfer occurred on day three or five and surplus embryos were frozen.

Sample size and statistical analysis

There were no published data from appropriately designed studies that provided estimated size effects on which to base a power calculation. Therefore, we designed this study with an *a priori* sample size of convenience [26] that was sufficient to detect an absolute difference in CPR of 25%, from 20% in the placebo group to 45% with melatonin treatment with a power of 80% at a level of 0.05. Using a placebo:intervention allocation ratio of 1:3 allowed us to explore possible dose effects of melatonin, requiring a minimum of 160 women (40 in the placebo group and each of the three treatment groups). While the 25% difference in pregnancy rate is relatively large, similar effect sizes have previously been used in other drug trials.[27] We acknowledge that the relatively small group size (n=40) means that this study is under-powered to detect smaller differences. Nonetheless, our study design usefully informs future larger trials of melatonin in IVF, not only with respect to expected size effects and therefore sample size required but also regarding doses of melatonin, if any, to study.

Where participants were cancelled before their EPU, their number of oocytes and embryos were entered as zero. Analyses for all dichotomous categorical variables were performed

using Chi-square or Fisher's exact tests where appropriate. Normally distributed continuous data was analysed across the four groups using ANOVA, otherwise the non-parametric equivalent was used (Kruskal-Wallis). Within-patient changes, were compared using paired samples t-tests and Wilcoxon-signed rank tests. Melatonin concentrations were analysed with ANOVA using log-transformed data. Trends across dosing groups for categorical and continuous clinical outcomes were assessed using Chi-square for trend and Spearman's rank correlation coefficient respectively. Trends across groups for count variables were analysed using Poisson regression.

As planned *a priori*, prespecified subanalyses were performed by combining treatment groups and comparing outcomes with placebo using Mann-Whitney-U and linear regression. To determine effects of cancelled cycles, primary outcome analyses were repeated after excluding these cases.

An independent interim analysis of safety data only (cancelled cycle rate, preclinical pregnancy loss and medication side effects) was performed by the DSMB at 50% recruitment, with stopping rules defined prior to this assessment.[24]

Statistical analysis was performed using SPSS v22.0 (IBM, Armonk, New York). P <0.05 was considered statistically significant for the primary outcome. For *a priori* determined secondary outcomes, we set the level of statistical significance at a more conservative level of p<0.005 to control for multiple comparisons. ITT results have been reported unless otherwise stated.

Ethics

This study was registered with the ANZCTR (Project ID: ACTRN12613001317785) with the protocol published before commencement of recruitment.[24] Ethics and Institutional Review Board approval was obtained from the Monash Health HREC (Project number: 13402B), Monash Surgical Private Hospital HREC (Project number: 14107), Monash University HREC

(Project number: CF14/523 - 2014000181), and Epworth HealthCare HREC (Project number: 634-34).

Results

Demographics

There were no differences at baseline for demographic variables (Table 3.1). Thirty women (19%) were aged ≥40 years and were evenly distributed across the groups. The ten participants who withdrew did not differ from those that were included in the analysis.

Compliance

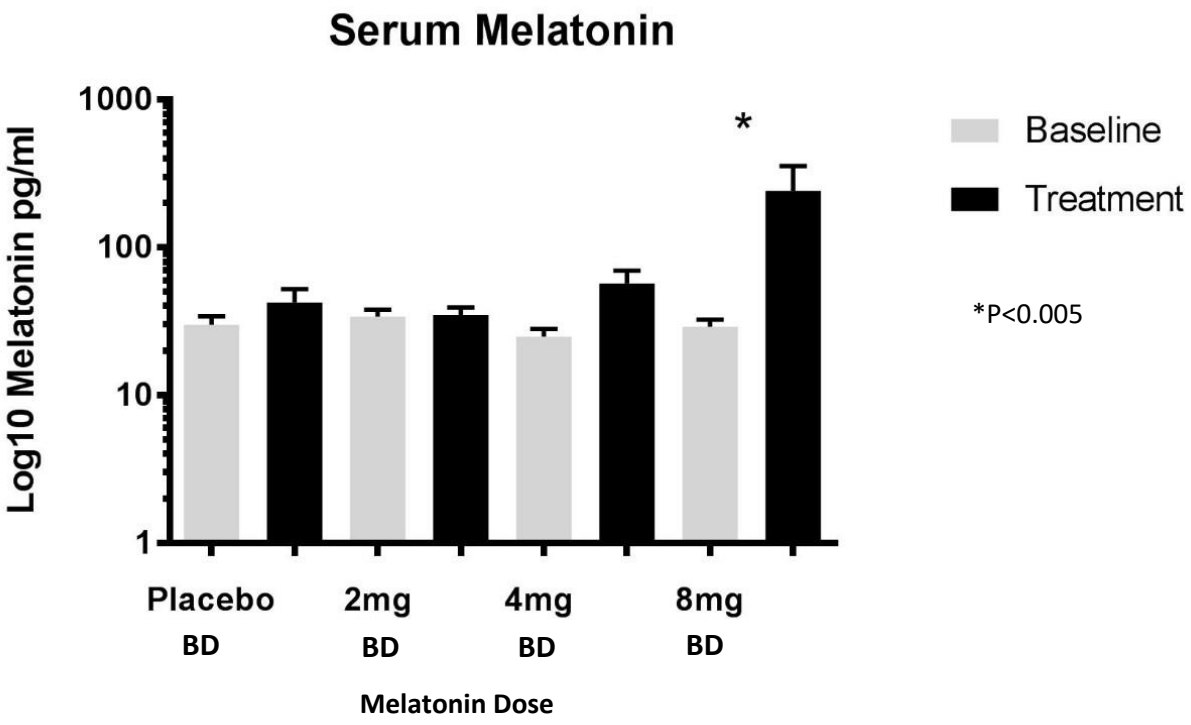
Compliance across groups was over 95%. The number of tablets and duration of intake did not differ between groups.

Serum and FF melatonin concentrations

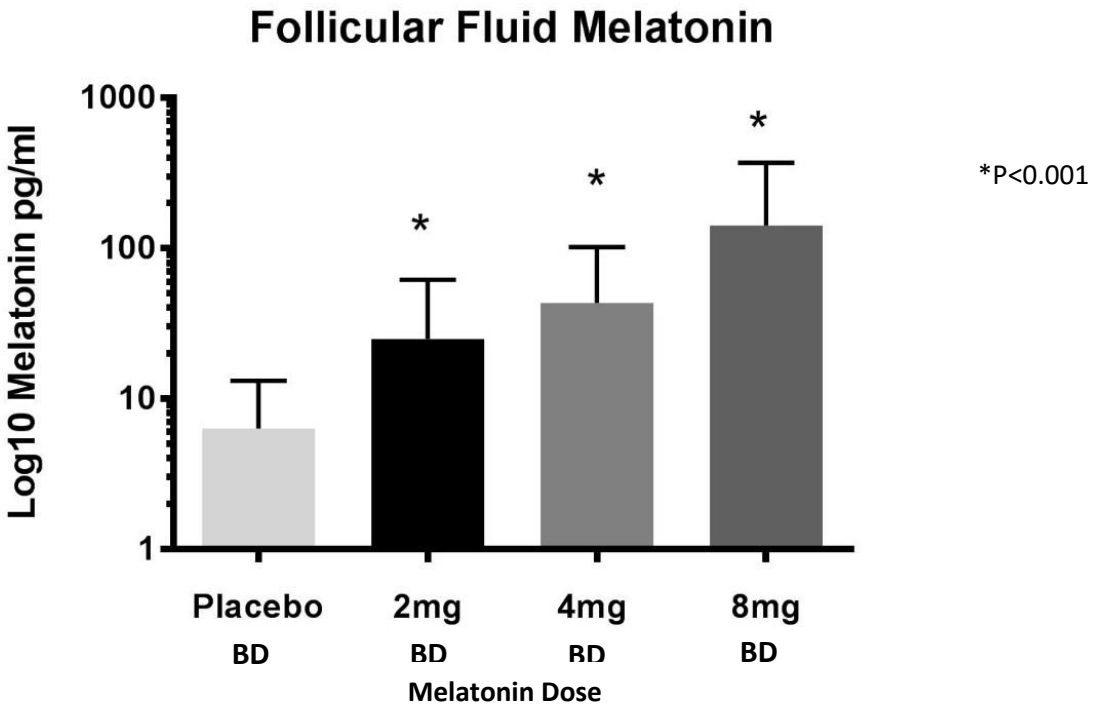
EPU occurred 14.2±1.7 hours after the last dose of trial medication and increasing doses of melatonin resulted in a dose-dependent increase in both serum and FF concentrations of melatonin (Figure 3.3A and 3.3B). Compared with placebo, FF melatonin concentrations increased by 3-fold, 6-fold and 9-fold for our three doses.

FIGURE 3.3 A) SERUM CONCENTRATIONS OF MELATONIN; B) FOLLICULAR FLUID CONCENTRATIONS OF MELATONIN

A)



B)



BD: twice daily

Serum oestradiol and progesterone concentrations

There were no statistically significant differences or dose-dependent effect on day eight to nine serum concentrations of oestradiol, progesterone or LH (Table 3.2) across all groups or when treatment groups were combined and compared with placebo. There was no statistically significant difference or dose-dependent effect on FF concentrations of oestradiol and progesterone (Table 3.2).

TABLE 3.2: SEX-STEROID CONCENTRATIONS AT BASELINE, DAY 8-9 AND AT OOCYTE RETRIEVAL (MEDIAN, IQR)

	Placebo BD N=36	Melatonin 2mg BD N=38	Melatonin 4mg BD N=36	Melatonin 8mg BD N=40	P value[a]	P value for trend[b]	Any melatonin	P value[c]
SERUM								
Baseline								
Oestradiol (pmol/L)	242 (152 – 414)	388 (189-612)	333 (162.5-464)	357 (225-549)	0.3	0.2	355 (187-524)	0.1
Progesterone (nmol/L)	11.6 (1.0-33.9)	19.8 (2.5-36.6)	11.9 (1.05-44.7)	23.7 (5.4-28.5)	0.7	0.5	18.1 (3.3-35.3)	0.4
Day 8-9								
Oestradiol (pmol/L)	2309 (1310 – 3079)	2264 (984 – 4231)	2355 (1193 – 4456)	2178 (1051 – 3666)	0.9	0.8	2249 (1152 – 4129)	0.8
Progesterone (nmol/L)	1.5 (0.9-2.2)	1.0 (0.8-1.6)	1.4 (0.9-2.5)	1.0 (0.7-1.9)	0.1	0.2	1.1 (0.8-2.0)	0.1
LH (IU/L)	1.2 (1.0-2.2)	1.4 (0.0-2.6)	1.6 (0.0-2.5)	1.0 (0.0-2.3)	0.8	0.4	1.3 (0.0-2.3)	0.6
Day of EPU								
Oestradiol (pmol/L)	2287 (1755-3045)	2642 (1705-3447)	1819 (1593-3205)	1985 (1337-3040)	0.4	0.3	2131 (1624-3277)	0.8
Progesterone (nmol/L)	23.8 (18.3-33.3)	22.0 (16.9-35.1)	21.6 (17.4-27.1)	21.4 (12.5-32.9)	0.8	0.3	21.7 (16.6-32.1)	0.4
FOLLICULAR FLUID								
Oestradiol (nmol/L)	851 (530-1290)	705 (425-1190)	629 (377-1115)	708 (517-1180)	0.9	0.4	702 (425-1178)	0.3
Progesterone (µmol/L)	25.8 (18.8-32.9)	22.2 (15.3-33.6)	25.0 (18.4-30.4)	30.4 (14.9-35.5)	0.6	0.8	25.0 (17.0-33.9)	0.7

[a]Kruskal Wallis; [b]Spearman-rank correlation; [c]Mann Whitney U; BD: twice daily

Oocyte number and maturity and embryo number and quality

There was no statistically significant difference between the groups for total oocyte number (p=0.8) or for the number of oocytes that were fertilised (p=0.6). There was also no significant dose-dependent trend in any oocyte or embryo parameter (Table 3.3). When assessing the number of MII oocytes (data was available for ICSI patients, N=96), there was no significant difference in the median number of MII oocytes between the groups (p=0.4).

There were no statistically significant differences in median number of embryos or quality of embryos across all groups or when comparing placebo with any dose of melatonin (Table 3.3).

TABLE 3.3: EMBRYO AND OOCYTE OUTCOMES

Treatment arm	**Placebo BD N=40**	**Melatonin 2mg BD N=41**	**Melatonin 4mg BD N=39**	**Melatonin 8mg BD N=40**	**P value[a]**	**IRR (95% CI)[b]**	**P value for trend across groups[b]**	**Any Melatonin N=120**	**P value[c]**
Number of oocytes	8·0 (3·25-13·0)	9·0 (0·0-16·0)	6·0 (0·0-14·0)	7·5 (3·25-11·0)	0·8	0.97 (0.92, 1.01)	0.2	8·0 (3·0-14·0)	0·4
Number of Fertilised oocytes	3·0 (1·0-6·0)	4·0 (0·0-7·5)	2·0 (0·0-5·0)	4·0 (1·25-6·0)	0·6	1.00 (0.93, 1.07)	0.9	4·0 (1·0-7·0)	0·8
Number of embryos	3·0 (1·0-5·75)	4·0 (0·0-7·5)	2·0 (0·0-5·0)	4·0 (1·25-6·0)	0·6	1.00 (0.93, 1.07)	0.9	3·0 (0·0-7·0)	0·9
Number of embryos utilised	2·0 (0·25-3·0)	1·0 (0·0-3·0)	1·0 (0·0-2·0)	2·0 (0·25-3·0)	0·7	0.94 (0.85, 1.03)	0.2	2·0 (0·0-3·0)	0·4
Number of good embryos (A and B)	1·5 (0·0-3·75)	2·0 (0·0-5·0)	2·0 (0·0-4·0)	2·0 (0·0-3·75)	0·9	1.00 (0.91, 1.07)	0.7	2·0 (0·0-4·0)	0·8
Number of poor embryos (C D and X)	1·0 (0·0-2·0)	0·0 (0·0-3·0)	0·0 (0·0-1·0)	1·0 (0·0-3·0)	0·1	1.03 (0.91, 1.05)	0.7	0·5 (0·0-0·5)	0·4

[a]Kruskal Wallis; [b]Poisson regression; [c]Mann-Whitney U, comparison between placebo and any melatonin; IRR: incidence rate ratio; Cancelled cycles and withdrawn patients considered as having zero oocytes and embryos; Results reported as Median (IQR) unless otherwise stated; X=zygotes discarded Day 3

Clinical pregnancy, live birth, biochemical pregnancy and adverse events

Neither the BPR (p=0.8), CPR (p=0.7), LBR (p=0.7) nor the rate of cancelled cycles before EPU (p=0.3) showed a dose-response relationship between groups (Table 3.4). There were no clinical pregnancies in the 30 women aged ≥ 40 years. The CPR for all the women who took melatonin (all three groups combined) was higher than those taking a placebo, but this did not reach statistical significance (21.7% vs 15.0%, OR 1.57 (95% CI 0.59, 4.14), p=0.4, absolute risk reduction (ARR) +6.7% (95% CI -6.6%, +20.0%)). This result did not differ significantly in the PP analysis (22.8% vs 16.7%, OR 1.48 (95% CI 0.56, 3.94), p=0.4, ARR +6.1% (95% CI -8.3%, +20.5%)). We performed logistic regression, including age as the only covariate found to effect CPR in a univariate analysis, and this did not significantly change the result significantly (adjusted OR 1.73 (95% CI 0.62, 4.78, p=0.3).

TABLE 3.4: CLINICAL OUTCOMES

	Placebo BD N=40	Melatonin 2mg BD N=41	Melatonin 4mg BD N=39	Melatonin 8mg BD N=40	P value	P value for trend across groups	Any Melatonin N=120	OR (95% CI)	P value[c]
Cancelled cycle before EPU (%)	6/40 (15.0)	12/41 (29.3)	10/39 (25.6)	6/40 (15.0)	0.3[a]	0.3[b]	28/120 (23.3)	1.73 (0.66, 4.53)	0.3
Cancelled cycle between EPU and ET (%)	7/40 (17.5)	2/41 (4.9)	9/39 (23.1)	7/40 (17.5)	0.1[a]	0.5[b]	18/120 (15.8)	0.83 (0.32, 2.17)	0.6
Biochemical pregnancy rate per cycle started (%)	8/40 (20.0)	14/41 (34.1)	7/39 (17.9)	9/40 (22.5)	0.3[a]	0.8[b]	30/120 (25.0)	1.33 (0.55, 3.21)	0.5
CPR per cycle started (%)	6/40 (15.0)	11/41 (26.8)	6/39 (15.4)	9/40 (22.5)	0.5[a]	0.7[b]	26/120 (21.7)	1.57 (0.59, 4.14)	0.4
CPR per ET (%)	6/27 (22.2)	11/27 (40.7)	6/20 (30.0)	9/27 (33.3)	0.5[a]	0.6[b]	26/74 (35.1)	1.90 (0.68 – 5.29)	0.2
CPR per ET* (%)	8/29 (27.6)	11/28 (39.3)	9/25 (36.0)	9/30 (30.0)	0.8[a]	0.9[b]	29/83 (34.9)	1.75 (0.64 – 4.81)	0.3
LBR per cycle started (%)	6/40 (15.0)	11/41 (26.8)	6/39 (15.4)	9/40 (22.5)	0.5[a]	0.7[b]	26/120 (21.7)	1.57 (0.59, 4.14)	0.4
LBR per ET (%)	6/27 (22.2)	11/27 (40.7)	6/20 (30.0)	9/27 (33.3)	0.5[a]	0.7[b]	26/74 (35.1)	1.90 (0.68 – 5.29)	0.2
LBR per ET* (%)	8/29 (27.6)	11/28 (39.3)	9/25 (36.0)	9/30 (30.0)	0.8[a]	0.9[b]	29/83 (34.9)	1.75 (0.64 – 4.81)	0.3
Mean birthweight (SD)	3240 (228)	3249 (168)	3267 (227)	3492 (186)	0.8[d]	0.6[e]	3337 (342)	1.00 (0.99, 1.00)	0.7[d]
Gestation ≥37 weeks	5 (83·3)	10 (90·9)	6 (100·0)	8 (88·9)	0·6[a]	0.8[b]	24 (92·3)	2.40 (0.18, 31.88)	0.5

*These include the first frozen embryo transfer of patients having an elective 'freeze all' cycle; [a]Chi-square; [b]Chi-square for trend across treatment groups; [c]Chi-square, comparison between placebo and any melatonin; [d]Estimated marginal means; [e] Spearman-rank correlation coefficient = 0.1; ET: embryo transfer; EPU: oocyte retrieval; CPR: clinical pregnancy rate; LBR: live birth rate; Patients who were randomised but did not take trial medication were coded as 'cancelled cycle before EPU'

Of all 160 patients who were randomised, 59 (36.9%) did not reach ET in their first cycle. Ten (17%) were withdrawn prior to commencing trial medication (Figure 3.1), 20 (35%) had unexpected poor ovarian response to stimulation, 14 (24%) were cancelled after EPU but before ET because of a lack of transferable embryos, 11 (19%) required a 'freeze all' cycle, 2 (3.4%) had a premature LH surge and 2 (3.4%) experienced an error in stimulation medication administration. For the ITT analysis, patients who were recruited but withdrawn were coded as 'cancelled before EPU'. Therefore, the cancellation before EPU rate in the women taking melatonin (all doses) was 23.3% compared with 15.0% for those taking placebo (OR 1.73 (95% CI 0.66, 4.53), p=0.3). There were no significant differences in clinical outcomes following PP analysis, however, the rate of cancelled cycles before EPU was 19.3% in the any melatonin group, compared with 5.6% in the placebo group (OR 4.07 (95% CI 0.91, 18.22), p=0.07).

The rate of preclinical pregnancy loss did not differ between groups, although the total number of preclinical pregnancy losses was small (n=6). There were no ectopic pregnancies. One woman, in the 2mg BD melatonin group, had a term livebirth of a baby with an absent right kidney. One baby from the placebo group was born weighing 1300g at 29 weeks and no babies in the melatonin groups were born <2500g. One patient each in the 2mg BD and the 8mg BD melatonin groups gave birth between 34 and 37 weeks. One patient each in the 4mg BD and the 8mg BD melatonin groups were diagnosed with preeclampsia and there was one case of major placenta praevia in the 2mg BD melatonin group.

'Minor' adverse effects were reported as 'none' by 33.3% of women in the placebo group and by 28.1% in the melatonin groups (p=0.6). The most commonly reported adverse effect was headache, reported by 50% of women in the placebo group and by 45% of those taking any dose of melatonin (p=0.6). The rates of fatigue also did not differ between placebo and melatonin arms (16.7% vs 28.1%, p=0.2). A detailed analysis of sleep outcomes is presented elsewhere.[28]

Subanalyses

To assess the effect of cancelled cycles before EPU, we performed a subanalysis excluding these patients. We found no differences in oocyte or embryo parameters and no difference in CPR (28.3% vs 17.6%, OR 1.84 (95% CI 0.68, 4.96), p=0.2).

In PP analysis of CPR and LBR (excluding women who withdrew prior to commencing trial medication), there were no significant differences when any melatonin dose was compared with placebo (22.8% vs 16.7%, OR 1.48, 95% CI 0.56, 3.94, p=0.4). To assess the outcome of the 'first transfer', including those who did not have a transfer in their first cycle due to a 'freeze all' cycle (N=11), we assessed CPR and LBR including the first frozen embryo transfer in these patients (Table 3.4). The CPR and LBR was not significantly different between any melatonin and placebo groups (34.9% vs 27.6%, OR 1.75, 95% CI 0.64, 4.81, p=0.3).

Discussion

We designed this pilot clinical trial to determine the effectiveness of melatonin on IVF outcomes and, if appropriate, to inform sample size calculations and a target melatonin dose for future clinical trials. In our study, oral melatonin during ovarian stimulation, up to 16mg daily, did not statistically significantly improve outcomes from IVF.

Our interest in melatonin as a method of improving IVF success was primarily due to its potent effects as an antioxidant, as opposed to any presumed effects on sleep. Melatonin is unique in the family of antioxidants for several reasons. It is a suicidal terminal antioxidant, with its metabolites also acting as antioxidants,[29] it is amphiphilic, allowing it to gain access to intra- and extra-cellular targets; it acts via receptors but also directly on free radicals; and it has the ability to potentiate the actions of other endogenous antioxidants.[3] Melatonin has a relatively short half-life.[23] It is likely that a single daily dosing regimen, such as those tested

previously, would not achieve sustained antioxidant effects during the IVF cycle. This may, at least in part, explain why previous studies exploring the efficacy of melatonin in improving IVF success have been inconclusive.[30] For this reason, we chose to examine a more frequent dosing regimen, twice per day, together with higher doses.[23]

FF melatonin and sex steroids

Serum and FF concentrations of melatonin and sex steroids were measured so that we would be able to assess biochemical changes in relation to clinical outcomes. Our dosing regimens resulted in measurable differences in concentrations of circulating and FF melatonin. In that regard, we observed an increase in FF concentrations of melatonin of 3-fold, 6-fold and 9-fold for our three doses, compared with placebo. The absolute concentrations are dissimilar to those observed by Tamura and colleagues, however, the relative increase compared with controls was similar between our studies (3-fold vs 4-fold).[4] Therefore, the difference in absolute concentrations are most likely a reflection of the different assay techniques used.

There was no difference in FF concentrations of oestradiol and progesterone between the placebo and melatonin groups, suggesting that melatonin is not significantly associated with the synthesis of these hormones in the follicle. This is in contrast to previous findings in swine models.[31]

Oocyte and embryo outcomes

Based on the findings of previous reports, we also assessed the impact of melatonin on oocyte and embryo number and quality. We found no apparent effects on number or quality of either oocytes or embryos.

In a recent retrospective analysis, Tong and colleagues[32] identified that higher endogenous FF melatonin concentrations are correlated with a higher response to ovarian stimulation. While a moderate correlation existed, the lack of molecular pathway analysis or controlling

for other factors associated with ovarian response makes it difficult to conclude that melatonin deficiency causes poor ovarian response.

Our findings also contrast with those of others, all of which have used oral melatonin given during ovarian stimualtion.[3, 5, 17, 18] Eryilmaz and colleagues studied 60 women with sleep disorders and unexplained infertility.[17] They found that a single 3mg dose of melatonin given at night almost doubled the number of oocytes retrieved, from 6.9 to 11.5 ($p<0.001$) and the number of MII oocytes (4.0 to 9.0, $p<0.001$), and increased the proportion of grade 1 embryos from 45% to 69% ($p=0.05$). However, while that trial was randomised it was not blinded and introduced further bias by excluding cancelled cycles from analysis. The authors were also unable to control for possible confounding factors such as number of previous failed IVF cycles and parity. Another study that used patients as their own controls after an unsuccessful non-melatonin IVF cycle also found an increase in the proportions of fertilised oocytes and good quality embryos in their subsequent melatonin cycle.[33] The within-subjects design limits the reliability of the conclusions drawn by this study because of the phenomenon of regression to the mean.[34]

In keeping with our findings, Batioglu and colleagues[5] found that, when comparing controls with melatonin, the total number of oocytes retrieved and the number of mature oocytes did not differ (10.9 ± 4.0 vs 12.0 ± 6.0, $p=0.14$). This study excluded cancelled cycles. In our study, we focussed on first cycles, controlled for multiple demographic variables, and were able to assess multiple doses of melatonin. In our sub-analysis, after excluding patients who had their cycles cancelled before EPU, there was no significant difference between groups in any oocyte or embryo parameters, although the difference widened. It is therefore probable that previous reports of improvements in embryological outcomes after melatonin treatment that have excluded cancelled cycles have overestimated the benefit of melatonin in these circumstances. Our inability to confirm measurable effects on gametes and embryos

with any dose casts significant doubt on the plausible mechanisms by which melatonin might improve LBR.

Clinical pregnancy, live birth rates and adverse events

This study is the first to assess the LBR in women taking melatonin during their first IVF cycle. Despite achieving a dose-dependent increase in FF melatonin concentrations, none of our analyses demonstrated an increase in CPR or LBR compared with placebo. This is in keeping with the absence of any measurable effects on egg or embryo number or quality and largely in accord with previous studies that have also failed to show an improvement in pregnancy rate.[4, 5, 17] A recently published randomised trial assessing the combined use of melatonin with myo-inositol specifically in participants with polycystic ovarian syndrome (PCOS) has also found no significant improvement in CPR.[35]

There were no significant differences in maternal side effects, adverse pregnancy outcomes or fetal abnormalities between the placebo and treatment arms. We have previously reported the lack of adverse effects on daytime alertness and sleep quality, which is reassuring in view of the twice daily melatonin administration.[28]

Cancelled cycles

The number of recruited women who did not reach fresh ET was 59 (36.9%). This was largely due to the number of cancelled cycles before EPU. In our PP analysis (excluding women who were withdrawn), there was a non-statistically significant increase in cancelled cycles before EPU when comparing any dose of melatonin with placebo (19.3% vs 5.6%, OR 4.07 (95% CI 0.91, 18.22), p=0.07). This result needs to be interpreted with caution because of the wide confidence interval. Nevertheless, we cannot exclude the possibility that melatonin may cause a reduction in the success of ovarian stimulation.

Strengths and Limitations

This is the first dose-finding double-blind placebo-controlled randomised trial assessing the effect of melatonin on LBR in women undergoing their first IVF cycle. A further key strength is the analysis of circulating and FF melatonin concentrations following the administration of three doses of melatonin administered twice daily to promote sustained antioxidant concentrations.

Our study failed to detect a statistically significant difference in CPR per cycle started between placebo and treatment groups. We were also able to follow up the outcome of the first frozen embryo transfer from patients who had a 'freeze all' cycle. Even after inclusion of these, pregnancy rates were no different between groups. We recognise that this study lacks power and while we have not shown a statistically significant difference in our primary outcome, this does not mean that a smaller clinically significant difference does not exist. Based on the LBR per cycle started (following the first transfer of a fresh or frozen embryo) in this trial (placebo: 8/40 [20.0%]; all melatonin groups: 29/120 [24.2%]) a future study would need to recruit approximately 3100 patients. About 7200 eligible patients would need to be approached based on our recruitment rate of 371 eligible patients over two years.

Furthermore, we were unable to determine the outcomes of all embryos that were frozen from a trial cycle as some of the MIART embryos remain in cryopreservation. As such, we cannot exclude that cumulative pregnancy rates from a melatonin treatment cycle may be higher than in the placebo group. However, this appears unlikely given that the total number of useable embryos was not significantly different between the groups.

It can also be argued that the maximal benefit of an adjuvant therapy such as melatonin would be experienced by patients who are 'poor responders'. While some of the women included in this trial were aged 40 years or over, we cannot exclude that melatonin has a

potential beneficial effect in poor responders as no clinical pregnancies were recorded in these women.

Finally, the CPR in our placebo group is low (15%) but the 95% confidence interval around this point estimate suggests that the true rate is between 5.7% and 29.8%. It is likely that a repeat trial would show a regression to the mean with a higher pregnancy rate in the placebo group rendering the difference between the placebo and treatment groups even smaller.

Conclusion

In this pilot clinical trial, we have shown that while oral melatonin in high doses can result in supraphysiological serum and FF concentrations, there is insufficient evidence to suggest melatonin improves CPR after IVF in women undergoing their first cycle of treatment. We also failed to demonstrate a difference in secondary outcome measures, including the number of mature oocytes and embryo quality, and have not been able to find any dose-dependent effects on clinical outcomes. Recognising that our study lacked power for the primary outcome, the observed effect size in this pilot dose-finding study could inform the design of a larger randomised controlled trial of melatonin in IVF. However, as we have demonstrated no differences in oocyte or embryo outcomes and no dose-dependent clinical effects, the plausibility of a positive effect of melatonin on CPR remains questionable.

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Author contributions

SF, EMW and LR contributed to inception, concept and design, acquisition of data, data analysis and interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article. BV, ML, NH, MW, AL, CR, KL, PT contributed to acquisition of data, drafting of the manuscript, critical revision of the manuscript and approval of the article.

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Chapter Four

Ovarian blood flow and follicular development

4.1 THE EFFECT OF MELATONIN ON ULTRASOUND MARKERS OF FOLLICULAR DEVELOPMENT

Together with being a potent oxygen scavenger, melatonin is known to influence blood flow in several vascular beds. Blood flow to the reproductive organs, in particular the ovaries, may have an influence on reproductive health and fertility (Dickey, 1997, Razik, et al., 2015). Three-dimensional (3D) Power Doppler is emerging as a potential method for imaging the blood supply to the ovaries and ovarian follicles, and may represent a novel way of predicting IVF success.

Despite the lack of improvement in clinical outcomes shown in *Chapter Three*, the possibility that melatonin could influence subtle stimulation parameters without influencing clinical pregnancy rate remains. If melatonin had any effect on ultrasound markers of ovarian response, this may provide clues concerning dosing regimens, timing and duration of dosing that may then inform future studies.

In order to determine whether patients who were participating on the MIART trial experienced a change in blood flow to their ovaries while taking their trial medication, and to see whether these indices could predict IVF outcomes, I performed an analysis of day 6-10 transvaginal ultrasound results on a subset of these women.

4.2 THE EFFECT OF MELATONIN ON ULTRASOUND MARKERS OF FOLLICULAR DEVELOPMENT: A DOUBLE-BLIND PLACEBO-CONTROLLED RANDOMISED TRIAL

Abstract

Purpose

Melatonin is gaining increasing popularity as anti-oxidant adjuvant therapy for women undergoing in-vitro fertilization (IVF). It is also capable of altering blood flow in various vascular beds. While melatonin is largely thought to exert a beneficial effect on IVF success rates via its anti-oxidant capabilities, its effect on gonadal blood flow has never been previously assessed. We aimed to determine the effect of melatonin on ovarian vascular indices during ovarian stimulation for IVF. In addition, we wished to determine whether these indices could be used to predict IVF outcomes.

Materials and Methods

A double-blind placebo-controlled randomised trial. Sixty-nine women from the 'Melatonin in Assisted Reproductive Technology (MIART)' trial, undergoing their first cycle of IVF were randomised to receive either identical looking placebo (n=21), 2mg (n=13), 4mg (n=17) or 8mg (n=18) of melatonin, twice a day. Each participant underwent a transvaginal ultrasound at day 6-10 assessing follicular number and size. Additionally, the vascularisation index (VI), flow index (FI) and vascularisation-flow index (VFI) were measured in each ovary. These indices were then correlated with oocyte and embryo parameters.

Results

There were no differences in baseline characteristics between groups. The median number of follicles >11mm in diameter did not differ between groups. There were no statistically significant differences in the VI, FI or VFI in the right ovary or the FI or VFI in the left ovary between groups. When comparing placebo to any dose of melatonin, there was no

statistically significant difference in any measured parameter. The inter- and intra-observer reliability of the ovarian vascular indices was moderate to good. While there was correlation between the number of follicles on ultrasound and all measured embryological outcomes, there was no correlation between ovarian vascular indices and these important clinical outcomes.

Conclusion

Melatonin, in the doses tested, does not change ovarian vascular indices during ovarian stimulation. In addition, such vascular indices cannot predict the number or quality of oocytes or embryos obtained in an IVF cycle.

Keywords: Melatonin, assisted reproductive technology, IVF, ultrasound, power Doppler

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Introduction

Traditionally the ovarian response to hormonal stimulation prior to assisted reproductive technology (ART) has been measured biochemically, by serial oestradiol concentrations, together with standard two-dimensional (2D) ultrasound determination of follicular number and size [1]. Information obtained from these follicular phase ultrasounds are used to make decisions regarding whether or not to continue the cycle and also when the ideal time is to trigger follicular maturation. Unfortunately, while providing useful insights into ovarian

response and follicular number, traditional 2D ultrasound measurements do not provide insights to either follicular or oocyte quality. Such insights would be likely to assist in the prediction of the ultimate success of the assisted reproductive procedure. As ultrasound technology has improved, the usefulness of newer, non-invasive measurement techniques, such as 3-dimensional (3D) power Doppler (PD), have begun to be evaluated [2].

Promising results have indicated that follicular power Doppler studies are positively correlated with ovarian response and other markers of ovarian reserve, but not necessarily with clinical pregnancy after IVF/ET [3], and not in women with polycystic ovarian syndrome [4].

Others have focussed on the measurement, by 3D PD, of vascularity index (VI), flow index (FI) and vascularisation flow index (VFI) [5], VI estimates the number of vessels in the measured volume, FI estimates the average flow rates through these vessels by measuring the intensity of the colour output and VFI is an aggregate measure of the two [5,6]. However, the utility of these measures in predicting ART success is still under-investigated and uncertain.

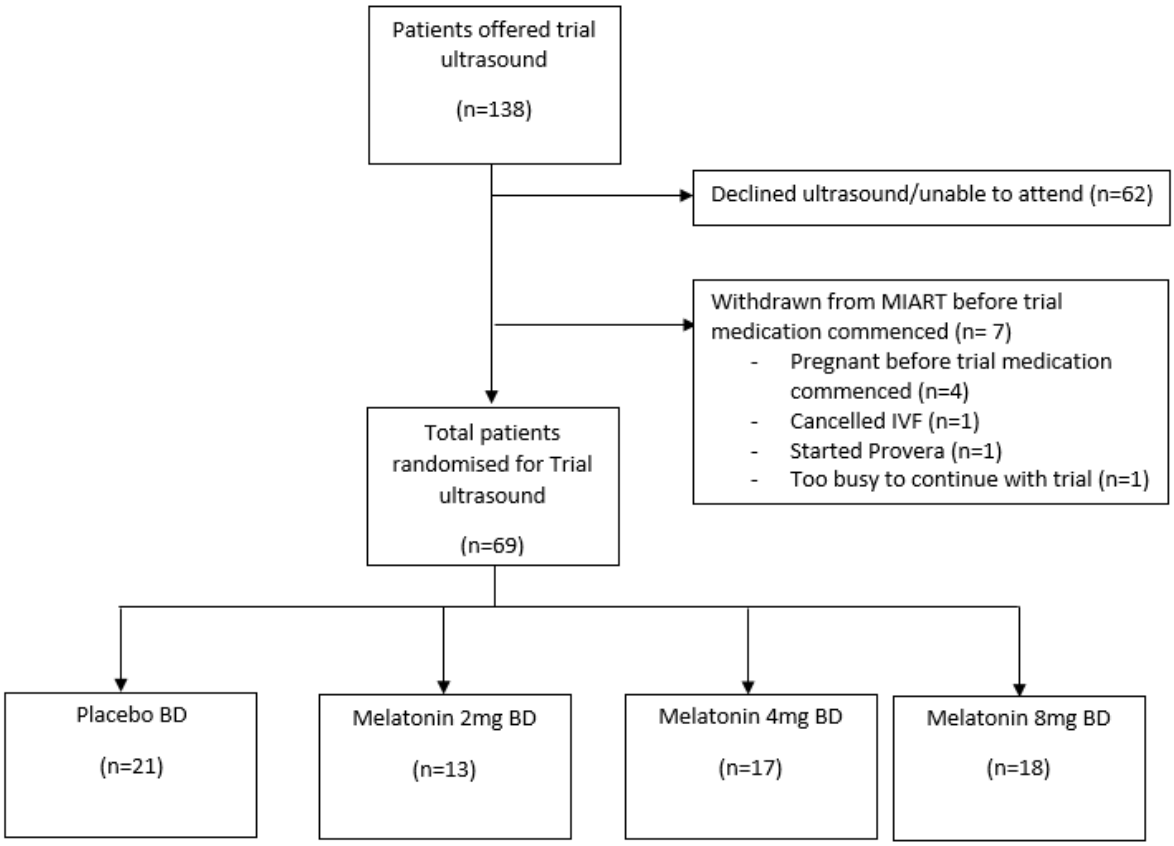
There has been growing interest in the use of the potent oxygen scavenger, melatonin, as a means of improving IVF success rates. One proposed mechanism of action is that due to its variable effect on human vasculature [7], melatonin may have a beneficial effect on blood flow to the ovaries. Considering this possibility, we used a subset of patients recruited for the 'Melatonin in Assisted Reproductive Technologies (MIART) trial' (ACTRN12613001317785) [8], to determine whether the administration of different doses of melatonin can change the blood flow to the ovaries during gonadotrophin stimulation and to assess whether these indices are predictive of oocyte and embryological outcomes in this cohort.

Methods

Because of the pilot nature of this study and to provide clarification for future design of larger randomised trials, a convenience sample of 69 participants was obtained by offering participants of the MIART trial inclusion in the ultrasound arm of the study. These participants were recruited from Monash IVF centres in Melbourne, Australia between September 2014 and September 2016. Inclusion criteria are outlined elsewhere [8]. These participants were offered additional measurements at their routine Day 6-10 follicular ultrasound scan. Of 138 participants, 62 declined participation in this arm, because of an inability to attend the trial ultrasound scan. Seventy-six were consented and randomised using the minimisation method by the factors age, body mass index (BMI), parity and smoking status [9]. After randomisation, 7 patients withdrew from the MIART trial before commencing the trial medication.

Therefore, 69 participants were available for intention-to-treat (ITT) analysis: placebo (n=21), 2mg (n=13), 4mg (n=17) and 8mg (n=18) of melatonin (Figure 4.1).All patients took the allocated trial medication twice per day (between 0800 and 1000 and between 2000 and 2200) from Day 2 of their cycle until the night before egg collection.

FIGURE 4.1: RECRUITMENT FLOWCHART



BD: twice per day; IVF: in-vitro fertilization; MIART: Melatonin in Assisted Reproductive Technology trial; mg: milligrams

This study was double-blinded, with allocation known only by the prescribing pharmacy and an independent party. Routine 2D ultrasound with a 6-12MHz transvaginal transducer was used to take grayscale measurements of follicular development at Day 6-10, together with endometrial appearance and thickness, ovarian volume and follicular number >11mm, which were counted manually. In addition, 3D power Doppler was used to measure ovarian VI, FI and VFI.

All measurements were taken by one of two highly trained sonographers using a transvaginal probe on a Voluson E8 Expert, Software EC250 copyright 2013 General Electric (GE Healthcare Austria GmbH & Co OG, 2013) using Vocal II software (GE healthcare, Austria, 2004) and analysed by a specialist gynaecological sonologist. All women were examined in the lithotomy position after emptying their bladder.

Ovarian volume was determined using the methods described by Makled et al [10], and Jokubkiene et al [11] using Virtual Organ Computer-aided Analysis (VOCAL). In the 3D ultrasound mode with power Doppler, a longitudinal section of the ovary was obtained and a 3D power Doppler ultrasound data set was obtained. The setting “Quality mid 2” was chosen as the sweep speed for the 3D ultrasound. After recording of each volume, the resultant multiplanar display was examined to ensure that a complete volume of the ovary had been captured. Volumes were transferred to a server for storage in a database for later analysis. Ovarian vascular indices (VI, FI and VFI) were then determined offline using the 4D-view™ software, version 9.1 (GE Medical Systems, Zipf, Austria) with the virtual organ computer-aided analysis (VOCAL™) software. All calculations were performed on images of the ovary using the midsagittal view as the reference image and recording in the longitudinal, transverse and coronal plane. Based on the findings of Jokubkiene and associates [11], we chose a rotation step of 30 degrees for all images. The outline of each ovary was traced manually in all planes, and VI, FI and VFI were calculated.

Tests for intra-observer variation were performed on a subset of 14 (21.7%) participants chosen at random from the sample with the same specialist taking two measurements from the same ovaries in two separate sittings. Separate tests for inter-observer error were also performed for these participants with another specialist sonographer blinded to the findings of the other sonologist.

Embryological outcomes were determined by skilled blinded embryologists. Oocytes were scored for maturity. Embryos were scored as 'good', being a grade of 'A' or 'B'; or 'poor', representing grades of 'C' or 'D'. Clinical pregnancy was defined as the presence of a live intrauterine pregnancy at 7-week ultrasound.

All statistical analyses were performed using SPSS v22 (IBM, Armonk, New York) with the intention-to-treat principle. Baseline demographics were compared for similarity using ANOVA for normally distributed continuous data and Chi square with Fishers exact test where required for categorical data. Vascular indices were compared between groups using Kruskal-Wallis for non-parametric data and ANOVA for normally distributed data. Dose-effects were analysed using chi-square for trend (categorical variables) and Spearman's rank correlation for continuous outcomes. Intraclass correlation coefficients were determined for the intra-observer (One-way random, consistency, single measures) and inter-observer (Two-way mixed, consistency, single measures) analysis. Spearman correlation analysis was performed to assess the relationship between vascular indices and the total number of oocytes, MII oocytes and embryos. To control for the large number of outcomes assessed and considering that these were *a priori* determined secondary outcomes of the MIART trial, a more conservative level of significance was chosen ($p<0.005$).

Human research ethics approval was obtained from the Monash Health HREC (Project number: 13402B), Monash Surgical Private Hospital HREC (Project number: 14107), Monash

University HREC (Project number: CF14/523 - 2014000181), and Epworth HealthCare HREC (Project number: 634-34). Participants provided written informed consent.

Results

Sixty-four participants were available for analysis. There was no significant difference in baseline demographics between the groups (Table 4.1). Fifteen (21.7%) women were at least 40 years or older, and these were evenly distributed across the groups.

TABLE 4.1: BASELINE CHARACTERISTICS

	Placebo BD N=21	Melatonin 2mg BD N=13	Melatonin 4mg BD N=17	Melatonin 8mg BD N=18	Any melatonin N=48
Age Mean(SD)*	34.5 (4.5)	35.2 (4.5)	36.9 (3.7)	36.7 (4.6)	36.4 (4.2)
BMI Mean (SD)*	24.0 (4.7)	24.5 (4.6)	25.9 (4.4)	24.0 (4.3)	24.8 (4.4)
Smoker	2 (9.5)	0 (0.0)	0 (0.0)	1 (5.6)	1 (2.1)
Parity N (%)					
0	18 (85.7)	11 (84.6)	13 (76.5)	16 (88.9)	40 (83.3)
≥1	3 (14.3)	2 (15.4)	4 (23.5)	2 (11.1)	8 (16.7)
Type of ART					
IVF, n (%)	8 (38.1)	7 (53.8)	7 (41.2)	9 (50.0)	23 (47.9)
ICSI, n (%)	13 (61.9)	6 (46.2)	10 (58.8)	8 (44.4)	24 (50.0)
Both IVF and ICSI	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6)	1 (2.1)
Aetiology					
Endometriosis	3 (14.3)	1 (7.7)	1 (5.9)	3 (16.7)	5 (10.4)
PCOS	1 (4.8)	0 (0.0)	1 (5.9)	1 (5.6)	2 (4.2)
Tubal	3 (14.3)	3 (23.1)	2 (11.8)	3 (16.7)	8 (16.7)
Male factor	4 (19.0)	4 (30.8)	5 (29.4)	5 (27.8)	14 (29.2)
Idiopathic	11 (52.4)	6 (46.2)	10 (58.8)	8 (44.4)	24 (50.0)
Day of scan					
6-7	0 (0.0)	0 (0.0)	2 (11.8)	2 (11.1)	4 (8.5)
8-10	21 (100.0)	13 (100.0)	15 (88.2)	16 (88.9)	44 (91.7)

BMI: body mass index; ART: assisted reproductive technology; IVF: in vitro fertilization; ICSI: intra-cytoplasmic sperm injection

To assess the reliability and reproducibility of ovarian vascular indices, intra and inter-observer measurements were performed on a random sample of 14 participants. We found moderate to good agreement for all indices for intra-observer analyses (Intraclass correlation (ICC) of 91-97%, $p<0.001$) (Table 4.2) and a wider range for inter-observer analysis (ICC 84-97%, $p<0.001$) (Table 4.3) [12].

TABLE 4.2: INTRA-OBSERVER DIFFERENCES: SONOGRAPHER 1

	Pass 1 Mean (SD)	Pass 2 Mean (SD)	Cronbach's Alpha	Intraclass correlation	95% CI	F	P value
Left VI	5.59 (4.02)	5.82 (3.64)	0.99	0.97	0.91-0.99	70.24	<0.001
Left FI	33.91 (4.83)	34.72 (4.07)	0.97	0.93	0.80-0.98	28.15	<0.001
Left VFI	2.02 (1.87)	2.07 (1.64)	0.98	0.97	0.89-0.99	56.50	<0.001
Right VI	4.97 (2.33)	4.99 (2.25)	0.97	0.95	0.85-0.98	36.89	<0.001
Right FI	33.57 (3.87)	34.16 (5.04)	0.96	0.91	0.76-0.97	21.75	<0.001
Right VFI	1.69 (0.82)	1.74 (0.83)	0.95	0.91	0.76-0.97	21.95	<0.001

Comparisons between 14 subjects; VI: vascularity index; FI: flow index; VFI: vascularity flow index

TABLE 4.3: INTER-OBSERVER DIFFERENCES: SONOGRAPHER 1 VS SONOGRAPHER 2

	Sonographer 1 Mean (SD)	Sonographer 2 Mean (SD)	Cronbach's Alpha	Intraclass correlation	95% CI	F	P
Left VI	5.59 (4.02)	5.43 (4.33)	0.986	0.97	0.91-0.95	71.05	<0.001
Left FI	33.91 (4.83)	33.09 (3.92)	0.913	0.84	0.56-0.95	11.53	<0.001
Left VFI	2.02 (1.87)	1.92 (1.94)	0.99	0.98	0.94-0.99	100.12	<0.001
Right VI	4.97 (2.33)	5.01 (2.45)	0.96	0.93	0.79-0.98	25.97	<0.001
Right FI	33.57 (3.86)	33.41 (3.50)	0.94	0.89	0.68-0.96	16.37	<0.001
Right VFI	1.69 (0.82)	1.65 (0.84)	0.95	0.91	0.74-0.97	20.40	<0.001

Comparisons between 14 subjects; VI: vascularity index; FI: flow index; VFI: vascularity flow index

The left sided VI was statistically higher in the 4mg BD group compared with the 8mg BD group (Median (IQR): 6.3 (5.3-10.4) vs 3.6 (1.4-6.0), $p<0.01$). There were no other statistically significant differences in VI, FI or VFI in either laterality or between any other groups (Table 4.4).

TABLE 4.4: ULTRASOUND PARAMETERS INCLUDING VASCULAR INDICES

	Placebo BD N=21	Melatonin 2mg BD N=13	Melatonin 4mg BD N=17	Melatonin 8mg BD N=18	P value between groups	P value for trend§	Any Melatonin N=43	P value
Left VI	4.8 (3.6-8.3)	4.5 (3.4-5.8)	6.3 (5.3-10.4)	3.6 (1.4-6.0)	0.03	0.2	4.6 (2.8-7.6)	0.5
Left FI	33.8 (32.2-36.6)	36.7 (32.0-40.4)	34.5 (33.1-37.1)	33.5 (29.1-36.3)	0.3	0.6	34.5 (31.6-37.9)	0.6
Left VFI	1.7 (1.2-3.1)	1.6 (1.2-2.4)	2.1 (1.8-3.9)	1.8 (0.6-2.6)	0.3	0.7	1.8 (1.0-2.8)	0.9
Right VI	5.6(3.2-7.8)	4.5 (3.1-6.3)	4.2 (2.9-5.4)	5.3 (2.8-6.9)	0.4	0.3	5.0 (3.0-6.1)	0.1
Right FI	34.6(31.6-36.9)	34.8 (30.5-38.5)	32.1 (29.1-34.9)	32.7 (30.1-34.4)	0.1	0.04	32.7 (30.3-35.6)	0.1
Right VFI	2.0 (1.1-2.9)	1.7 (1.1-3.9)	1.3 (0.9-1.9)	1.9 (1.0-2.3)	0.3	0.4	1.7 (1.0-2.3)	0.3
Total follicles >11mm	6.0 (4.0-10.5)	6.0 (1.5-9.0)	5.0 (2.0-8.0)	6.0 (1.0-8.0)	0.9	0.4	6.0 (2.0-8.0)	0.4
LOV (ml)	19.3 (10.4-21.6)	18.2 (9.3-24.0)	10.4 (6.9-16.6)	11.7 (7.6-18.4)	0.3	0.1	12.0 (7.7-22.2)	0.3
ROV (ml)	17.8 (11.4-29.4)	20.4 (9.5-27.2)	11.1 (6.4-27.7)	13.1(8.7-23.7)	0.3	0.1	14.4 (8.1-23.7)	0.5
ET Mean (SD)*	9.1 (2.8)	8.9 (2.1)	8.6 (2.6)	8.9 (2.6)	0.9	0.8	8.8 (2.4)	0.6
EA‡								
Non-specific, N(%)	2 (9.5)	1 (7.7)	1 (5.9)	1 (5.6)	1.0	0.7	3 (6.3)	1.0
Proliferative, N(%)	19 (90.5)	12 (92.3)	16 (94.1)	17 (94.4)			45 (93.8)	

All results are presented as Median (IQR) unless otherwise stated; Kruskal Wallis performed unless otherwise stated; *ANOVA – these variables are normally distributed; ‡Fishers exact; § Spearman correlation; VI: vascularity index; FI: flow index; VFI: vascularity flow index; LOV: left ovarian volume; ROV: right ovarian volume; ET: endometrial thickness; EA: endometrial appearance

There were no statistically significant differences in 2D measures of stimulation response, including the total number of follicles, follicles>11mm, endometrial thickness or ovarian volume between groups. When combining all melatonin groups and comparing with placebo, there were no significant differences in these parameters (Table 4.4).

The embryological outcome data of all patients were pooled and a Spearman correlation analysis was performed assessing oocyte and embryo outcomes. As expected, the total number of follicles on ultrasound was positively correlated with the number of oocytes (Spearman's rho = 0.68, $p<0.001$, N=64), number of MII oocytes (Spearman's rho = 0.28, $p<0.05$, N=64) and number of embryos (Spearman's rho = 0.62, $p<0.001$, N=64). However, there was no correlation between any ovarian vascular index and total number of oocytes, MII oocytes, fertilised oocytes, embryos or embryos utilised (Table 4.5).

There were 4 miscarriages in this cohort (6.3%), two in the placebo group and two in the 2mg BD melatonin group. There were 6 clinical pregnancies (9.4%), three in the placebo group, one in the 2mg BD melatonin group and two in the 4mg BD melatonin group. All clinical pregnancies resulted in live births.

TABLE 4.5: CORRELATION BETWEEN OVARIAN VASCULAR INDICES AND OOCYTE AND EMBRYO OUTCOMES: ALL GROUPS COMBINED

	Total follicles	**Total oocytes retrieved**	**MII oocytes**	**Fertilised oocytes**	**Total embryos**	**Poor quality embryos**	**Good quality embryos**	**Utilised embryos**
Median (IQR)	5.5 (2.0-8.8)	8.0 (3.0-14.0)	5.0 (2.0-7.0)	3.0 (0.0-6.0)	3.0 (0.25-6.0)	0.5 (0.0-2.0)	2.0 (0.0-3.8)	1.0 (0.0-2.8)
RVI^	0.02, 0.86	0.09, 0.46	0.01, 0.99	-0.08, 0.56	-0.09, 0.50	-0.02, 0.89	-0.02, 0.87	-0.12, 0.36
RFI^	-0.05, 0.69	0.03, 0.84	0.03, 0.79	0.09, 0.49	0.07, 0.59	0.15, 0.23	0.06, 0.62	0.06, 0.65
RVFI^	-0.16, 0.22	-0.06, 0.67	-0.04, 0.76	-0.19, 0.14	-0.18, 0.16	-0.02, 0.87	-0.14, 0.27	-0.24, 0.06
LVI^	0.12, 0.35	0.15, 0.24	-0.06, 0.66	0.11, 0.41	0.08, 0.56	0.05, 0.71	0.07, 0.57	-0.04, 0.79
LFI^	0.04, 0.74	-0.02, 0.88	0.04, 0.74	0.06, 0.66	0.04, 0.75	0.06, 0.64	0.01, 0.95	-0.04, 0.74
LVFI^	0.04, 0.79	0.03, 0.83	0.04, 0.79	-0.02, 0.91	-0.06, 0.66	-0.04, 0.77	-0.05, 0.67	-0.17, 0.19
Total follicles^	-	0.68, <0.001*	0.28, 0.03*	0.63, <0.001*	0.76, <0.001*	0.42, <0.001*	0.54, <0.001*	0.51, <0.001*

^Spearman correlation coefficient (rho,p) presented unless otherwise stated; IQR: inter-quartile range; RVI: right vascularity index; RFI: right flow index; RVFI: right vascularity-flow index; LVI: left vascularity index; LFI: left flow index; LVFI: left vascularity-flow index

Discussion

We have shown that increasing melatonin doses, at least in the range 2mg-8mg twice daily, has no apparent effect on ovarian blood flow. In addition, we have shown that ovarian vascular indices do not correlate with the number of oocytes or the embryo utilization rate.

Melatonin is a potent oxygen scavenger and has been shown to have effects on the vascular system in both animals and humans. This effect is mediated through stimulation of the melatonin receptors, MT1 and MT2 on the smooth muscle cells of blood vessels. Interestingly, the effects of melatonin on the vasculature appears to be dose-mediated, with effects not necessarily being directly proportional to the dose [13]. Vascular smooth muscle MT1 activation has been shown to result in vasoconstriction through its effects on noradrenaline pathways [14], and the stimulation of MT2 has been shown to result in vasodilation [13,15]. Vasodilation can also occur without melatonin receptors being involved [16]. This direct action of melatonin on vasculature together with the difference in effect that occurs as a result of the activation of melatonin receptors might, at least in part, account for the variable effect on the human vasculature that has also been demonstrated [7]. It is, therefore, conceivable that different vascular beds contain a variable distribution of MT1 and MT2 receptors which might account for the variation in response to melatonin.

Ovarian blood flow changes during the menstrual cycle, with improvements in flow being observed from the day before ovulation [17]. Links have previously been drawn between high ovarian follicular blood flow and improved follicular development [18], with some suggestion that pregnancy rates are improved with well-perfused follicles [19,20]. Very high ovarian peak systolic velocity (PSV) and very low PSV are associated with better and worse success rates respectively [21].

Some investigators have found that VI, FI and VFI are not able to predict the difference between ‘poor ovarian responders’ and ‘normal’ responders to ovarian stimulation [22].

Engels et al performed a study of 79 intrauterine insemination (IUI) cycles measuring these ovarian blood flow indices. They found a significantly lower subfollicular VI and VFI in women who became pregnant in that cycle [6]. They argue that while this seems counterintuitive, it is in agreement with another study which investigated unstimulated cycles [23], and may indicate an inversely proportional relationship between follicular blood flow and follicular response, whereby a reduction in vascularisation might improve the response to ovarian stimulation [6]. We did not find this in our study, where ovarian vascular indices did not correlate with the number of follicles.

While our study was not powered to detect the effects of blood flow differences in relation to pregnancy rates, we did show that ovarian indices did not change with melatonin dose. This suggests that while melatonin may have an influence on the vascular system in other body systems, this does not apply to the ovaries in the IVF population, even when using doses four times the currently accepted 'standard' dose. In addition, we have shown that these ovarian vascular indices are not predictive of oocyte or embryo outcomes in our cohort.

While we did not find any change in blood flow or vascularisation in the ovaries, we were not adequately powered to detect very small differences in ovarian vascular indices. However, the clinical significance of such small differences is uncertain. Furthermore, a sample of only 14 patients was used to compare reliability of ovarian vascular indices, and only two observers were tested. This may result in inaccuracies in reporting of reproducibility of measurements, and cannot account for larger variations in investigators. In addition, using the same dataset for analysis and for determination of intra- and inter-observer variation might overestimate the results reported. The pregnancy rates in this cohort were relatively low. This might be a reflection of the high proportion of older women included in the analysis. However, our sample size did not allow us to assess these parameters as *a priori* outcomes.

Finally, because we investigated patients undergoing their first IVF cycle in the context of a clinical drug-trial, these results may not be generalizable to the 'standard' IVF population.

In this double-blind placebo-controlled randomised study, we have shown that melatonin does not affect ovarian flow indices. In addition, we have shown that these indices do not correlate with oocyte number or the embryo utilization rate. This suggests that the ovarian vascular indices of VI, FI and VFI do not, in their current state, provide useful clinical information predicting the success of an IVF cycle.

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Chapter Five

Melatonin and sleepiness

5.1 THE EFFECT OF MELATONIN ON SLEEP

The majority of this thesis has focussed on melatonin and the importance of its function as an anti-oxidant. However, melatonin is still most widely prescribed nocturnally for treatment of insomnia. While it is traditionally associated with sleep and plays an essential part in circadian rhythmicity, it is not required to be labelled with a sedation warning by the Therapeutic Goods Administration in Australia.

Naturally, when considering the use of doses as high as 8mg twice per day (16 mg per day), which is a dose that is eight-fold higher than that standardly used for the treatment of insomnia, some concern might be raised regarding its effect on daytime sleepiness. When the dose is administered in the morning, a regimen which has undergone limited testing previously, it is understandable if patients are reluctant to participate. Despite this speculation, there have been no previously reported studies designed to assess the effect of melatonin at twice daily dosing. Furthermore, none have tested different doses in a placebo-controlled and blinded fashion. Certainly, none have assessed this effect in the IVF population.

As has been shown in *Chapter Three*, high doses of melatonin are required if significant changes to serum and follicular fluid concentrations are to be achieved. If melatonin were to become a routine adjuvant to IVF treatment, its safety profile would first need to be elucidated, and its effect on daytime sleepiness and night time sleep quality further understood. In order to achieve this, all participants on the MIART trial were invited to complete a 'sleep diary' and 'alertness score and compliance diary' (*Appendix C*).

5.2 THE IMPACT OF MELATONIN ON THE SLEEP PATTERNS OF WOMEN UNDERGOING IVF: A DOUBLE BLIND RCT.

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ORIGINAL ARTICLE

The impact of melatonin on the sleep patterns of women undergoing IVF: a double blind RCT

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STUDY QUESTION: Does melatonin result in a dose–response effect on sleep quality and daytime sleepiness in women undergoing IVF?

SUMMARY ANSWER: Melatonin, even when given at high doses twice per day, does not cause significant daytime sleepiness or change night time sleep quantity or quality.

WHAT IS KNOWN ALREADY: Melatonin is being increasingly used as an adjuvant therapy for women undergoing IVF owing to its anti-oxidative effects. It is widely considered to be sedative but there are scant objective data on the effects of melatonin on sleep in the setting of IVF.

STUDY DESIGN SIZE, DURATION: The study was a double-blind placebo-controlled randomized trial of 116 women recruited between September 2014 and September 2016.

PARTICIPANTS/MATERIALS, SETTING, METHOD: Women who were undergoing their first cycle of IVF at private IVF centers were recruited into the RCT and randomized to receive either placebo, 2 mg, 4 mg or 8 mg of melatonin, twice per day (BD) from Day 2 of their cycle until the day before oocyte retrieval. Each participant wore an accelerometer that provides an estimate of sleep and wake activity for up to 1 week of baseline and throughout treatment (up to 2 weeks). They also kept sleep diaries and completed a Karolinska sleepiness score detailing their night time sleep activity and daytime sleepiness, respectively.

MAIN RESULTS AND THE ROLE OF CHANCE: In total, 116 women were included in the intention-to-treat analysis (placebo BD ($n = 32$), melatonin 2 mg BD ($n = 29$), melatonin 4 mg BD ($n = 26$), melatonin 8 mg BD ($n = 29$)). There were no significant differences in daytime Karolinska sleepiness score between groups ($P = 0.4$), nor was there a significant dose–response trend (β=0.05, 95% CI −0.22–0.31, $P = 0.7$). There were no differences in objective measures of sleep quantity or quality, including wake after sleep onset time, sleep onset latency, and sleep efficiency before and after treatment or between groups. There was an improvement in subjective sleep quality scores from baseline to during treatment in all groups, except 8 mg BD melatonin: placebo (percentage change −13.3%, $P = 0.01$), 2 mg (−14.1%, $P = 0.03$), 4 mg (−8.6%, $P = 0.01$) and 8 mg (−7.8%, $P = 0.07$).

LIMITATIONS, REASONS FOR CAUTION: As this was a subset of a larger trial, the melatonin in ART (MIART) trial, it is possible that the sample size was too small to detect statistically significant differences between the groups.

WIDER IMPLICATIONS OF THE FINDINGS: While this study suggests that melatonin can be used twice per day at high doses to achieve sustained antioxidation effects, with the reassurance that this will not negatively impact daytime sleepiness or night time sleep habits, the sample size is small and may have missed a clinically significant difference. Nevertheless, our findings may have implications not only for future studies of fertility treatments (including meta-analyses), but also in other medical fields where sustained antioxidation is desired.

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TRIAL REGISTRATION NUMBER: This trial was prospectively registered with the Australian New Zealand Clinical Trials Registry (Project ID: ACTRN12613001317785).

TRIAL REGISTRATION DATE: 27/11/2013

DATE OF FIRST PATIENT'S ENROLMENT: 1/9/2014

Key words: melatonin / sleep / infertility / ART / IVF / oxidative stress / antioxidant / oxygen scavenger

WHAT DOES THIS MEAN FOR PATIENTS?

This paper asks whether melatonin has an impact on sleep and sleepiness in women who are going through IVF. It reports on a trial carried out in Australia where women having IVF were given melatonin at different doses twice a day during the first part of their IVF cycle. Melatonin is sometimes given to women having IVF as one in three women with fertility problems experience disturbed sleep and it has been suggested this could have a negative impact on IVF outcomes. Melatonin is also used as an antioxidant for women having IVF with the aim of possibly improving egg quality.

Melatonin is not stored easily in the body, so the researchers gave some women higher doses of melatonin as they wanted to see if this would make a difference to their sleep at night, and if it might make them feel sleepy during the day. They found that even at the highest dose, the melatonin did not cause significant daytime sleepiness. They also found that melatonin had no impact on sleep quality, quantity or daytime drowsiness at any dose. Although the women's night time sleep quality improved, the same thing happened to women who were given a placebo rather than melatonin.

This was quite a small study but it does suggest that women could take melatonin for its antioxidant qualities without it making them sleepy in the daytime, but also suggests that melatonin itself is not linked to better sleep in women going through fertility treatment.

Introduction

Women undergoing IVF for the treatment of infertility have increased levels of stress and associated sleep disturbance (Bjornsdottir *et al.*, 2014; Sanford *et al.*, 2014; Shibata *et al.*, 2014; Vitiello *et al.*, 2014). Anxiety is heightened and quality of life reduced in infertile patients (Sezgin *et al.*, 2016; Vitale *et al.*, 2016, 2017a, 2017b; Borghi *et al.*, 2017). Indeed, more than one in three infertile women have disturbed sleep (Lin *et al.*, 2014). Sleep disturbance may interfere with fertility through effects on the hypothalamic–pituitary axis and gonadotrophin regulation (Kloss *et al.*, 2015) or on immunity (Vgontzas *et al.*, 2004; Irwin *et al.*, 2006). It has also been hypothesized that sleep disturbance may have a negative effect on IVF outcomes (Goldstein *et al.*, 2017).

Melatonin has long been used in the general population to manage sleep dysfunction and improve sleep quality. Traditionally, oral melatonin has been used to improve sleep disturbance and better regulate sleeping patterns, particularly in those with insomnia (Garfinkel *et al.*, 1995; Cardinali *et al.*, 2012; Goldman *et al.*, 2014). It is also commonly used in travelers to manage 'jet-lag' (Herxheimer and Petrie, 2002) and by shift workers, as a means of shortening sleep onset latency and facilitating sleep phase shift (Xiang *et al.*, 2015; Sadeghniiat-Haghighi *et al.*, 2016). While not required to be labeled as a sedative, exogenous melatonin is largely believed to facilitate planned sleep onset as well as contribute to circadian regulation.

However, largely because of its additional capabilities as a potent oxygen scavenger and antioxidant enzyme inducer (Reiter *et al.*, 2016), melatonin is being increasingly prescribed for women undergoing IVF, sometimes in combination with other antioxidants such as myo-inositol (Lagana *et al.*, 2017), with the aim of reducing oxidative damage, thereby improving oocyte quality and subsequent pregnancy rates (Fernando and Rombauts, 2014). Therefore, the potential utility of melatonin in IVF treatments is two-fold – it may have a beneficial effect on sleep, but also, because of its potent antioxidant properties, it may protect oocytes and embryos from oxidative damage during the IVF process.

Melatonin has a short half-life when administered as a small single dose per day, therefore serum concentrations of melatonin decline rapidly and are unlikely to result in sustained antioxidation (Waldhauser *et al.*, 1984).

In addition, higher doses of melatonin are known to result in more sustained serum concentrations (Waldhauser *et al.*, 1984). In order to assess the effect of sustained antioxidation on IVF outcomes, the melatonin in ART (MIART) trial (Fernando *et al.*, 2014) was designed to assess twice daily melatonin dosing (at doses as high as 8 mg twice per day) on clinical pregnancy rates after IVF. A subset of patients was also asked to participate in a nested study to determine whether melatonin given in this manner would alter either daytime sleepiness or night time sleep quality and quantity in women undergoing IVF. We report the sleep and sleepiness findings here.

Materials and Methods

Participants

The MIART trial was a double-blind, randomized placebo-controlled multicenter trial of melatonin in women undergoing IVF (ACTRN12613001317785) (Fernando *et al.*, 2014). Eligibility criteria are listed in Table I. We have reported the full trial protocol previously (Fernando *et al.*, 2014). Briefly, participants were recruited from Monash IVF clinics in Melbourne, Australia between September 2014 and September 2016. Randomization was performed by blinded investigators using the minimization method by the factors age, BMI, parity and smoking status (Altman and Bland, 2005). Additional demographic information collected included etiology of infertility and night shift work. One hundred and twenty women (30 in each group) who were enrolled in the MIART trial, received one of four oral medications (placebo, 2 mg, 4 mg or 8 mg of sustained-release melatonin (which looked identical) twice per day (BD)). Trial medication was self-administered between 08:00 and 10:00 and then again between 20:00 and 22:00 from the first day of FSH administration on Day 2 of their menstrual cycle until the night before oocyte retrieval or, in the event of cycle cancellation, on the day of cancellation, whichever came earlier (8–14 days). Participants for this nested study were followed up until the completion of their oocyte retrieval. Medication compliance was assessed by a diary, in which participants recorded the dates and times that trial medication was taken, and by counting the number of capsules remaining after the trial period. All participants and investigators were blinded to the melatonin dose until the conclusion of the trial.

Actigraphy

Sleep quantity and quality were assessed using the Phillips Actiwatch2® (Philips Respironics, Pittsburg, PA, USA), an accelerometer that provides an estimate of sleep and wake activity based on activity level thresholds. Participants were asked to wear the watch all day and night except while bathing or swimming. They were asked to wear the watch for up to one week before ovarian stimulation commenced (baseline) and for up to 16 days during their treatment cycle, until the day of oocyte retrieval (treatment).

Actiwatches® were worn on the non-dominant wrist and configured for individual patients using 1 min epochs (activity measurement intervals) and medium sensitivity (activity threshold = 40 activity counts). Sleep onset and offset was identified after 10 min of inactivity and activity, respectively (Ancoli-Israel *et al.*, 2015). An action marker button was pressed by the participant when she put the watch on, took it off, just before falling asleep and just before getting out of bed in the morning. This allowed for validation of diary and actigraphy recordings.

Actigraphy analysis was performed using the validated Phillips Respironics Actiware® System version 6 (Philips Respironics, Pittsburg, PA, USA) (Cellini *et al.*, 2013). The sleep period was defined as the period of time from when the participant went to bed until the time they got out of bed and was manually scored based on event marker and sleep diary reports.

Data were compared separately for weeknights (Sunday–Thursday) and weekend nights (Friday and Saturday) (Biggs *et al.*, 2016).

Sleep diary

Participants were asked to maintain a sleep diary during the baseline and treatment periods to record bed time, bed time activity, sleep time, wake time and pertinent subjective measures of sleep including a perceived sleep quality ranking (from 1 = 'very good' to 4 = 'very poor'). They were also asked to record daytime sleepiness using the Karolinska sleepiness score (ranging from 1 = 'extremely alert' to 9 = 'extremely sleepy/fighting sleep') (Reyner and Horne, 1998). To allow for comparisons of daytime sleepiness, participants were asked to report their Karolinska sleepiness score at midday every day while taking the trial medication (Reyner and Horne, 1998).

Of the 120 patients, four declined actigraphy (leaving 116 for the intention-to-treat (ITT) analysis) and a further three patients withdrew from the trial before commencing trial medication. Twenty-seven patients were removed for the per-protocol analysis because of incomplete data or if night shifts were worked during the study (Fig. 1). Complete data (including all subjective and objective measures) were available from 89 women (placebo BD, $n = 25$; melatonin 2 mg BD, $n = 22$; 4 mg BD, $n = 17$; 8 mg BD, $n = 25$) for per-protocol analysis (Fig. 1).

Outcome measures

The major concern for participants in the MIART trial was the effect of melatonin on their daytime sleepiness, therefore the primary outcome of the present study was a subjective measure of daytime sleepiness measured on the Karolinska sleepiness scale (Horne and Biggs, 2013). Secondary outcome variables included a subjective ranking of sleep quality, sleep onset latency (time between going to bed and first epoch of sleep), wake after sleep onset (WASO) expressed as both the number of awakenings and the total time awake during the night, total sleep time (time between first and last epoch of sleep, less total time awake after the first sleep epoch), and sleep efficiency (total sleep time/time in bed expressed as a percentage).

Statistical analysis

Statistical analysis was performed using SPSS version 22 (IBM, Armonk, NY, USA). All participants were analyzed in the group that they were originally assigned to. All data were checked for outliers and were normally distributed. Baseline categorical demographics were compared using Chi-square or Fisher's exact tests where required. Continuous data were compared using ANOVA. Daytime sleepiness scores and sleep quality scores were tested for normality and Student's *t*-tests were used to compare means between placebo and any dose of melatonin. Repeated measures ANOVA (group × time) was conducted to determine group differences in subjective measures of sleep parameters between baseline and treatment. Linear regression was used to assess subjective daytime sleepiness and night time sleep quality ratings for any dose–response relationship with increasing melatonin treatment.

Linear mixed model analyses were conducted to determine changes in actigraphy outcomes between groups over time. Actigraphy-measured sleep outcomes were the dependent variables. 'Time' (baseline versus treatment, or 'treatment status') was entered as the repeated measure. 'Group' was entered as the fixed effect and subject code as the random effect and the interaction of group by treatment status (Group × Time) was determined. As there were no baseline differences in covariates, such as age and BMI, none were entered into the model.

Table I Eligibility criteria for women undergoing IVF in a double blind RCT of the impact of melatonin on sleep patterns.

Inclusion criteria	Undergoing first cycle of IVF or ICSI Aged between 18 and 45 years BMI between 18 and 35 kg/m^2 Undergoing a GnRH antagonist cycle (without OCP or Provera scheduling)
Exclusion criteria	Current untreated pelvic pathology – moderate to severe endometriosis, submucosal uterine fibroids/polyps assessed by the treating specialist to affect fertility, pelvic inflammatory disease, uterine malformations, Asherman's syndrome and hydrosalpinx Currently enrolled in another interventional clinical trial Concurrent use of other adjuvant therapies (e.g. Chinese herbs, Co-enzyme Q10, acupuncture) Current pregnancy Malignancy or other contraindication to IVF Autoimmune disorders Undergoing PGD Hypersensitivity to melatonin or its metabolites Concurrent use of any of the following medications Fluvoxamine Cimetidine Quinolones and other CYP1A2 inhibitors Carbemazepine, rifampicin Zolpidem, zopiclone and other non-benzodiazepine hypnotics Inability to comply with trial protocol

OCP, oral contraceptive pill; CYP1A2, Cytochrome P450 1A2.

Sample size was limited by the MIART study and, for this reason, precision estimates have been included in the presentation of results where possible.

Analysis was performed using both per-protocol and ITT principles. Results presented are based on the ITT principle, including all 116 patients who received objective sleep monitoring, unless otherwise stated. A value of $P < 0.05$ was considered statistically significant.

Ethics

This study was registered with the Australian and New Zealand Clinical Trials Register (ACTRN12613001317785). Human research ethics approval was obtained from the Monash Health HREC (13402B), Monash Surgical Private Hospital HREC (14107), Monash University HREC (CF14/523-2014000181) and Epworth HealthCare HREC (634-34). Written informed consent was obtained from all participants prior to enrollment on the trial.

Results

There were no significant differences between the groups in baseline demographics including age, BMI, parity, night shift work, smoking status or etiology of infertility (Table II). Medication compliance was above 95% in each group with no statistically significant differences between groups. The mean (SD) number of days of baseline and treatment recording was 4.7 (2.3) and 10.4 (2.3), respectively, and did not differ between groups. There was no difference between groups in the proportions of weekend nights included (Table II).

Daytime sleepiness scores are summarized in Fig. 2. The Karolinska sleepiness scores were not available for three patients who withdrew prior to commencing trial medication (Fig. 1). In addition, three patients did not record their Karolinska sleepiness score, leaving 110 with recorded Karolinska daytime sleepiness scores. The mean (SD) Karolinska score for these women was 4.21 (1.54), indicating that most women scored as 'relatively alert' for daytime sleepiness. There were no statistically significant differences between groups ($P = 0.4$). There was also no evidence of a dose–response trend between escalating melatonin dose and daytime sleepiness ($\beta = 0.05$, 95% CI −0.22–0.31, $P = 0.7$) (Fig. 2). There was no difference when comparing any dose of melatonin with placebo (Mean difference −0.3, 95% CI −0.9–0.4, $P = 0.4$).

Table III summarizes sleep quality scores. There were no differences in sleep quality between groups and no dose–response trend for sleep quality during treatment ($\beta = 0.05$, 95% CI −0.02–0.13, $P = 0.2$). Sleep quality did improve significantly from baseline to during treatment in all groups, except 8 mg BD melatonin: placebo (percentage change −13.3%, $P = 0.01$), 2 mg (percentage change −14.1%, $P = 0.03$), 4 mg (percentage change −8.6%, $P = 0.01$) and 8 mg (percentage change −7.8%, $P = 0.07$).

Table IV shows the average objective measures of sleep parameters at baseline and during treatment. Women in all melatonin groups got out of bed significantly earlier than participants receiving placebo ($P < 0.05$). However, this did not follow a dose–response pattern. In addition, there were no significant differences in time to bed, time in bed, total sleep time, onset latency, sleep efficiency or WASO either between groups or from baseline to during treatment. There were also no dose-dependent effects from baseline to treatment.

In all analyses, there were no significant differences between the ITT and per-protocol analyses.

Discussion

Here we report sleep outcome data from the first double-blind placebo-controlled randomized trial designed to measure the impact of different doses of oral melatonin on IVF outcome. We have shown that at doses as high as 8 mg BD there is no effect of melatonin on either subjective measures of sleep quality or daytime sleepiness or on objective measures of sleep quality and quantity.

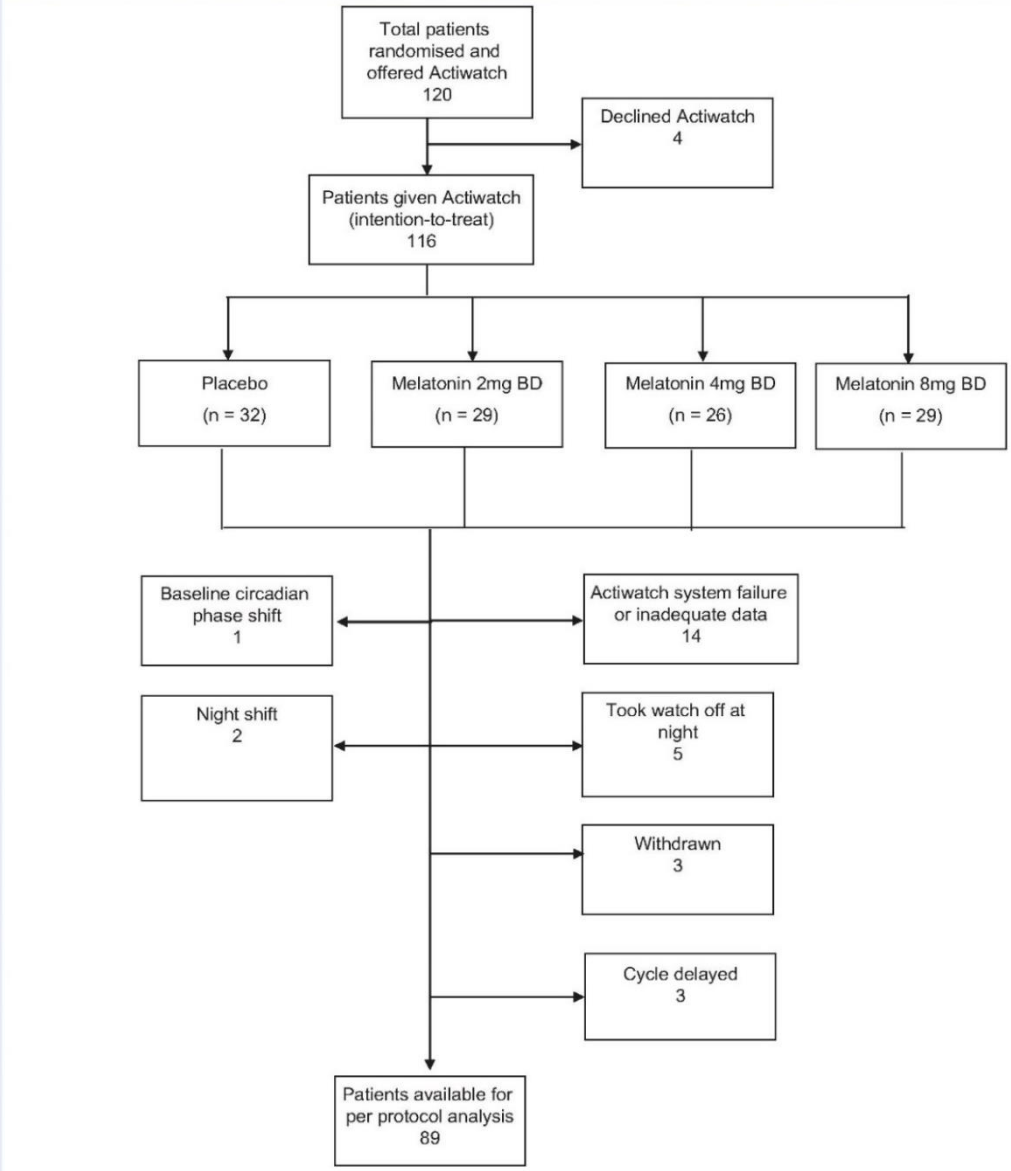

Figure 1 Recruitment flowchart for a RCT of the impact of melatonin on the sleep patterns of women undergoing IVF. Actiwatch is an accelerometer that provides an estimate of sleep and wake activity based on activity level thresholds. BD: twice per day.

A commonly used dose of melatonin during IVF treatment is 3–4 mg given once at night. Melatonin has a relatively short half-life (Waldhauser *et al.*, 1984). Given as a once per day dose, melatonin reaches peak serum concentrations within 1–3 h, which then decline over 8–12 h (Waldhauser *et al.*, 1984). It is likely that such a single daily dosing regimen does not achieve sustained antioxidant effects during the IVF cycle. This may explain why previous studies exploring the efficacy of melatonin in improving IVF success have been inconclusive (Gooneratne *et al.*, 2012). A more frequent dosing regimen, such as BD, would be expected to result in more sustained increases in systemic melatonin levels. This would, in turn, result in more sustained protection from oxidative stress. For this reason, in the MIART trial (Fernando *et al.*, 2014), we chose to examine administration BD in this dose-finding study, aiming to determine whether melatonin given in such a dosing regimen might improve

Table II Demographics of participants.

	Placebo BD $N = 32$	Melatonin 2 mg BD $N = 29$	Melatonin 4 mg BD $N = 26$	Melatonin 8 mg BD $N = 29$
Mean (SD) age (years)	35.3 (4.0)	35.0 (4.5)	35.8 (4.5)	35.9 (4.4)
Mean (SD) BMI (kg/m^2)	24.1 (4.6)	24.6 (4.0)	25.0 (4.8)	25.2 (4.0)
Parity				
0	25 (78.1)	25 (86.2)	21 (80.8)	22 (75.9)
≥1	7 (21.9)	4 (13.8)	5 (19.2)	7 (24.1)
Night shift work *N* (%)	1 (3.1)	1 (3.4)	2 (7.7)	2 (6.9)
Smoker *N* (%)	3 (9.4)	0 (0.0)	1 (3.8)	2 (6.9)
Etiology of infertility				
Endometriosis	4 (12.5)	5 (17.2)	5 (19.2)	2 (6.9)
PCOS	0 (0.0)	3 (10.3)	1 (3.8)	2 (6.9)
Tubal	6 (18.8)	6 (20.7)	3 (11.5)	2 (6.9)
Ovulatory	1 (3.1)	1 (3.4)	0 (0.0)	1 (3.4)
Male factor	5 (15.6)	11 (37.9)	7 (26.9)	12 (41.4)
Social	1 (3.1)	2 (6.9)	1 (3.8)	2 (6.9)
Idiopathic	16 (50.0)	9 (31.0)	14 (53.8)	10 (34.5)
Mean (SD) days of recording baseline*	4.0 (2.4)	4.7 (2.4)	4.9 (2.2)	5.3 (2.3)
Mean (SD) days of recording during treatment*	10.8 (1.9)	10.0 (2.3)	10.6 (2.9)	10.1 (2.2)
Friday or Saturday included in baseline *N* (%)*	14 (56.0)	16 (72.7)	16 (84.2)	22 (84.6)
Friday or Saturday included during treatment *N* (%)*	25 (100.0)	22 (100.0)	18 (94.7)	25 (96.2)
Sunday to Thursday included at baseline *N* (%)*	23 (92.0)	20 (90.9)	17 (89.5)	25 (96.2)
Sunday to Thursday included during treatment *N* (%)*	25 (100.0)	22 (100.0)	19 (100.0)	26 (100.0)

*Data available for 92 patients (89 with complete data and three with data eventually excluded: see Figure 1). All continuous variables reported as mean (SD); BD, twice per day; PCOS, polycystic ovary syndrome.

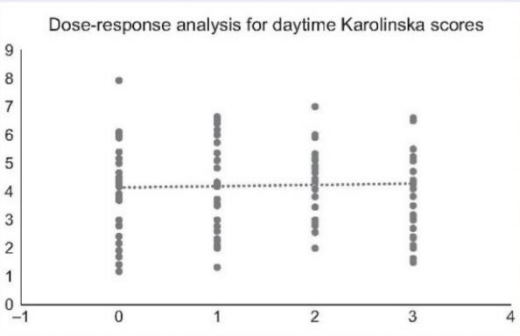

Figure 2 Dose–response analysis for daytime Karolinska sleepiness scores. There was evidence (n = 110) of a dose–response trend between increased melatonin dose and daytime sleepiness score (β=0.05, 95% CI −0.22–0.31, P = 0.7), and no differences in score when comparing any dose of melatonin with placebo (mean difference −0.3, 95% CI −0.9–0.4, P = 0.4).

IVF outcomes. As an *a priori* determined outcome, we also wished to explore the effect of such BD melatonin on sleep and daytime sleepiness because any beneficial effect on sleep in infertile women is likely to have a positive impact on patients (Goldstein *et al.*, 2017) and any negative effects on sleep quality or daytime sleepiness may restrict its use in future clinical trials, in this and other medical disciplines.

Several pharmacokinetic studies have addressed long-term night time dosing of melatonin as a treatment for sleep disorders in the elderly and infirm (Dawson *et al.*, 1998; de Castro-Silva *et al.*, 2010; Lemoine *et al.*, 2011; Gooneratne *et al.*, 2012). Such studies have tested doses ranging from 0.1 mg/nocte to 10 mg/nocte for up to 12 months (Zhdanova *et al.*, 2001; Bourne *et al.*, 2008). Only higher doses of melatonin resulted in a more persistently elevated circulating melatonin concentration and improved night time sleep (Zhdanova *et al.*, 2001). A recent meta-analysis of elderly patients with neurodegenerative disease found that melatonin improves subjective measures of sleep quality, as assessed by the Pittsburgh Sleep Quality Index, but has no effect on objective outcomes (Zhang *et al.*, 2016). For subjective outcomes, this meta-analysis included one trial with only eight participants. The effect of night time melatonin on sleep quality has also been examined in young people. In a trial of 21 adolescents who were given 1 mg melatonin in the evening, melatonin appeared to reduce daytime sleepiness and improve sleep quality (Eckerberg *et al.*, 2012). This trial did not assess morning doses of melatonin and the reduction in daytime sleepiness was attributed to improved sleep quality at night. In a meta-analysis of five studies involving 57 participants, Rossignol and colleagues (Rossignol and Frye, 2011) assessed the impact of melatonin (0.75–15 mg nocte for 14 days to 4 years) on the treatment

Table III Subjective measures of sleep quality between groups.

		Placebo BD N = 25	Melatonin 2 mg BD N = 26	Melatonin 4 mg BD N = 23	Melatonin 8 mg BD N = 26	β (95% CI)*	*P* for trend across groups*	Any Melatonin N = 75	Mean difference (95% CI)^	*P* value
Sleep quality score (mean+/−SD)	Baseline	2.25 (0.53)	2.21 (0.61)	2.22 (0.46)	2.23 (0.48)	−0.003 (−0.09–0.09)	0.9	2.22 (0.52)	0.03 (−0.21–0.26)	0.8
	Treatment	1.93 (0.49)	1.87 (0.45)	2.04 (0.38)	2.05 (0.40)	0.05 (−0.02–0.13)	0.2	1.99 (0.42)	−0.05 (−0.21–0.16)	0.6
Absolute change in sleep quality score (%)		−0.30 (−13.3)	−0.31 (−14.1)	−0.19 (−8.6)	−0.18 (−7.8)	0.05 (−0.04–0.14)	0.3	−0.22 (−9.9)	−0.08 (−0.32–0.16)	0.5
P value**		0.01	0.03	0.01	0.07	–	–	–	–	–

**Paired samples Student's *t*-test; *Linear regression for dose–response trend; ^Student's *t*-test. A lower score indicates better subjective sleep quality.

of sleep dysfunction in children with autism spectrum disorders. They found that night time melatonin improved sleep quality overall, increased sleep duration and shortened sleep onset latency. Taken together, these studies suggest that melatonin, when given at night, can be used to improve sleep. However, they provide no insights into the effect of melatonin on sleep and daytime sleepiness when the melatonin is given during the day.

In that regard, we found that daytime administration of melatonin, up to 8 mg BD, which would be considered a relatively high dose, affected neither subjective nor objective measures of sleep when compared with placebo. We did find that night time sleep quality significantly improved from baseline among almost all women, including women taking the placebo. The only group of women not to show a statistically significantly improved sleep quality was the group taking the highest dose of melatonin (8 mg twice daily). The reason for apparently improved sleep quality among the other three groups of women, including those taking the placebo, is not immediately apparent and was not expected. It is possible that a Hawthorne effect, where participants 'respond' solely because they are aware that they are being studied (McCambridge *et al.*, 2014), was at play. It is also possible that the relief of starting IVF treatment, in the hope or expectation of success, improved sleep quality. Sleep quality varies across the menstrual cycle (de Zambotti *et al.*, 2015; Mehta *et al.*, 2015) and that might also explain our findings because, by necessity, we compared sleep between the menstrual phase and late follicular phase. However, all of these possible explanations would apply equally to all groups of women.

Because of its circadian effects, most studies of melatonin have been performed in a 'sleep' setting, and therefore, dosing has been nocturnal. In our study, melatonin was administered BD, with the intention of sustaining its antioxidant affect throughout the day, without limiting it to the circadian night. Despite high doses given in the morning and evening, melatonin did not increase daytime sleepiness. This suggests that melatonin can be administered, even at relatively high doses, during the day without effecting daytime alertness. Because of its short half-life (Waldhauser *et al.*, 1984), this would allow for a more sustained anti-oxidant effect throughout the circadian cycle. This increases the dosing possibilities for the use of melatonin as an antioxidant to treat oxidative stress-related medical conditions (Esteban-Zubero *et al.*, 2017; Martinez-Campa *et al.*, 2017), with the reassurance that morning dosing does not increase daytime sleepiness. However, these findings should first be confirmed in further studies in these clinical contexts.

The strength of our trial is its randomized double-blind design and inclusion of a placebo group. This is particularly important when assessing subjective outcomes. We also assessed the effects of three different doses of melatonin with a view to likely future dosing regimens. If melatonin is shown to be effective, whether in IVF or for other indications such as fetal neuroprotection or as an adjuvant therapy for pre-eclampsia (Hobson *et al.*, 2013; Biran *et al.*, 2014; Miller *et al.*, 2014), this study provides supportive evidence that melatonin (at the doses tested) is unlikely to affect sleep quality or quantity when used in the short term. The analysis and comparison of both subjective and objective measures of sleep also minimizes the likelihood of bias.

A limitation of this study was the small sample size. The number of women in each group was determined by pregnancy rate, the primary outcome of the MIART trial (Fernando *et al.*, 2014), rather than by expected differences in sleep quality. It is possible that the sample size

Table IV Average objective measures of sleep quality/quantity by group at baseline and during treatment.

	Placebo BD		Melatonin 2 mg BD		Melatonin 4 mg BD		Melatonin 8 mg BD		Group *F*-value	Time *F*-value	Time × Group *F*-value
	Baseline (*N* = 23)	Treatment (*N* = 25)	Baseline (*N* = 21)	Treatment (*N* = 22)	Baseline (*N* = 18)	Treatment (*N* = 19)	Baseline (*N* = 25)	Treatment (*N* = 26)			
Bed time#	21:33 (4:39)	21:28 (4:35)	22:31 (0:50)	21:17 (4:45)	19:02 (8:12)	20:16 (6:55)	20:32 (6:07)	21:19 (4:22)	1.10	0.05	0.56
Get up time#	7:09 (1:07)	7:16 (0:44)	7:30 (0:49)	7:20 (0:48)	7:59 (1:40)	7:28 (1:04)	7:19 (1:00)	6:46 (0:55)	2.12	7.83[a]	2.87
Time in bed (hours)	8:33 (1:15)	8:50 (1:06)	8:59 (1:00)	8:58 (0:34)	8:57 (1:15)	8:40 (0:42)	8:52 (1:00)	8:33 (0:43)	0.48	0.91	2.43
Total sleep time (hours)	6:57 (1:03)	7:04 (0:52)	7:18 (0:54)	7:20 (0:36)	7:03 (0:54)	6:52 (0:32)	6:54 (1:35)	7:00 (0:41)	1.03	0.01	0.38
Onset latency (mins)	20.4 (17.6)	22.5 (14.0)	22.1 (19.4)	19.6 (10.9)	33.5 (23.3)	28.7 (13.1)	21.9 (15.9)	21.6 (14.6)	2.62	0.34	0.63
Sleep efficiency (%)	81.6 (8.9)	80.4 (5.4)	81.5 (6.2)	81.9 (4.5)	79.3 (6.1)	79.3 (5.1)	77.7 (16.7)	81.9 (4.8)	0.63	0.53	1.24
WASO (mins)	51.3 (23.9)	53.6 (22.6)	54.8 (19.5)	57.0 (20.7)	60.6 (21.9)	58.6 (17.7)	59.4 (26.7)	52.2 (14.5)	0.52	0.38	1.56

Linear mixed model analysis showing differences between treatment groups (Group), between baseline and treatment status (Time), and the interaction of group by treatment status (Group × Time); WASO: Wake after sleep onset; sleep efficiency: total sleep time/time in bed; Bonferroni for all comparisons; mins: minutes; #24-hour clock; [a]$P < 0.05$.

was too small to detect statistically significant differences between the groups. We sought to address this by accounting for the inter-individual differences through the mixed model analysis. The size of this study limits generalizability of the results; however, the data presented here would be appropriate for inclusion in future meta-analyses.

Actigraphy has been described as a viable and practical alternative to polysomnography in monitoring sleep disturbance (Hyde *et al.*, 2007; Marino *et al.*, 2013; Meltzer *et al.*, 2012). While this has the advantage that measurements can be taken in an environment more indicative of actual sleep conditions and is economical and minimally labor intensive, it is limited because assumptions are drawn based on the level of movement and its association with sleep, with limitations being more obvious in its specificity and in the detection of wakefulness (Meltzer *et al.*, 2012; Horne and Biggs, 2013; Marino *et al.*, 2013). We sought to overcome these limitations by the use of the device in conjunction with a detailed sleep diary (Wolfson *et al.*, 2003) and by including a period of 'calibration' (de Castro-Silva *et al.*, 2010; Horne and Biggs, 2013).

In summary, in this small randomized trial, we have shown that daytime dosing of melatonin in doses of 2 mg, 4 mg or 8 mg BD does not appear to affect night time sleep quality or daytime sleepiness in the population studied. While these findings are reassuring for the use of daytime melatonin, whether in future clinical trials in assisted reproduction or other conditions that might benefit from melatonin's antioxidant actions (Hobson *et al.*, 2013; Miller *et al.*, 2014), the limitations associated with our sample size must be considered when considering such trials.

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Authors' roles

S.F., S.N.B., R.S.C.H., E.M.W. and L.R. contributed to inception, concept and design, acquisition of data, data analysis and interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article. B.V., M.L., N.H., M.W., A.L., C.R., K.L., P.T. contributed to acquisition of data, drafting of the manuscript, critical revision of the manuscript and approval of the article.

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Conflict of interest

S.F., E.W., R.H., B.V., N.L., N.H., M.W., M.L., A.L., P.T., K.L. have nothing to declare. L.R. is a Minority shareholder in Monash IVF Group has unrestricted grants from MSD®, Merck-Serono® and Ferring® and receives consulting fees from Ferring®. S.N.B. reports consulting fees from Johnson & Johnson Consumer Inc®, outside the submitted work.

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Chapter Six

Discussion and Conclusions

6.1 SUMMARY OF FINDINGS

Melatonin, a very potent oxygen scavenger, is gaining momentum as a potential adjuvant therapy in IVF. Because of its anti-oxidative effects, it is theorised to improve the quality of oocytes and embryos and, therefore, pregnancy rates after IVF.

The MIART trial, a double-blind randomised placebo-controlled dose-finding study, has found that oral melatonin when given at doses of 2mg, 4mg or 8mg twice per day during IVF stimulation, does not improve the number or quality of oocytes or embryos or clinical pregnancy rates. This is despite being able to demonstrate a significant dose-dependent increase in follicular fluid concentrations of melatonin (*Chapter Three*).

Chapter Four demonstrated that melatonin at the doses tested, also does not appear to alter blood flow to the ovaries or the uterus when given during ovarian stimulation. This important finding suggests that, together with a lack of clinical benefit, melatonin does not result in a subtle difference in vascular supply to key reproductive organs. In addition, melatonin does not increase the number or size of ovarian follicles.

This placebo-controlled double blinded randomised trial is the first to assess the effects of short-term oral melatonin on both subjective and objective measures of sleep in this population. Presented in *Chapter Five*, I have shown that even at twice daily high doses, melatonin does not increase daytime sleepiness or change night time sleep quality or quantity in this population. Interestingly, I have also shown that subjective night time sleep quality appears to improve from pre-treatment to during treatment. However, these differences occurred across all groups, including the placebo group. This may indicate an improvement in sleep quality that is attributable to psychological or physiological responses to the IVF process itself, and this may warrant further investigation.

The MIART trial has shown that melatonin in the doses tested does not change clinical pregnancy rate or cancelled cycle rate (*Chapter Three*), ultrasound markers of ovarian

response to stimulation (*Chapter Four*) or sleep and daytime sleepiness (*Chapter Five*). Unfortunately, being a small study, it lacked power to detect the primary outcome of clinical pregnancy rate, and as such, the conclusions of this trial are not definitive and the efficacy of melatonin on IVF outcomes still requires further investigation. However, these preliminary findings suggest that the use of melatonin in this context should be discontinued until larger trials can be performed to refute these findings.

6.2 STRENGTHS AND LIMITATIONS

The MIART trial is a small pilot trial. Because it lacked power to detect a small difference in clinical pregnancy rates, it cannot exclude that such a smaller difference does not exist. Despite this, the lack of effect on major clinical secondary outcomes (eg. Oocyte and embryo number and quality) suggests that a true positive effect of melatonin on clinical outcomes is unlikely. A much larger trial would be required to determine a benefit (or lack thereof) definitively.

Because of the lack of clinical difference demonstrated in this trial, concerns may be raised about the bioavailability of the compounded trial medication that was used. However, each trial medication was independently verified for content. Importantly, I was able to demonstrate a definitive dose-dependent increase in biochemical concentrations of melatonin in both serum and follicular fluid, showing that the administered trial medication was able to both increase serum concentrations and reach the target tissue in follicular fluid. This suggests adequate bioavailability in this context.

The MIART trial is limited in its generalisability in that I have only included patients having their first cycle of IVF. It is likely that choosing this relatively 'low risk' group may have impacted negatively on power. Therefore, it is possible that selecting a 'higher risk' group, such as those with 'poor ovarian response', may have led to significant results, and this cannot be excluded. Women undergoing their first cycle of IVF were selected because of the

relative lack of contamination in this group (no previous IVF cycles, no previous failed or successful cycles etc.) and also because of the known challenges recruiting patients with 'poor ovarian response' for a blinded placebo-controlled IVF trial. Therefore, the lack of effect that we have shown can only be generalised to women having their first cycle of IVF.

Interestingly, the cancelled cycle rate was high in the melatonin groups, and while this did not reach statistical significance when compared with placebo, this is the first suggestion that melatonin may not be entirely harmless. This was most marked in the per-protocol analysis, after exclusion of patients who were withdrawn from the trial prior to commencement of trial medication (19.3% vs 5.6%, OR 4.07 (95% CI 0.91, 18.22), p=0.07). This result should be interpreted with caution, as the cancellation rate before EPU of 5.6% in the placebo group was far lower than the baseline rate of cancelled cycles at Monash IVF during the trial period (11%), which may have artificially inflated the difference between groups in the per-protocol analysis.

Table 6.1 shows the breakdown of cancelled cycles by treatment group. While there was an absolute difference in the rates of poor stimulation between the placebo group and any melatonin group, this was not statistically significant (5.0% vs 15.0%, OR 3.35 (95% CI 0.74, 15.14, p=0.5)). While this might suggest that melatonin has a negative impact on ovarian stimulation, the numbers were small (as reflected by the wide confidence interval) and therefore, further large randomised trials are required to confirm this.

TABLE 6.1: BREAKDOWN OF CANCELLED CYCLES

	Placebo BD N=40	Melatonin 2mg BD N=41	Melatonin 4mg BD N=39	Melatonin 8mg BD N=40	P value	P value for trend across groups	Any Melatonin N=120	OR (95% CI)	P value[c]
Cancelled cycle before EPU (%)	6 (15.0)	12 (29.3)	10 (25.6)	6 (15.0)	0.3[a]	0.3[b]	28 (23.3)	1.73 (0.66, 4.53)	0.3
Poor stimulation	2 (5.0)	8 (19.5)	5 (12.8)	5 (12.5)	0.4[a]	0.6[b]	18 (15.0)	3.35 (0.74, 15.14)	0.1
Withdrawn	4 (10.0)	3 (7.3)	3 (7.7)	0 (0.0)	0.3[a]	0.1[b]	6 (5.0)	0.47 (0.13, 1.77)	0.3
Other[d]	0 (0.0)	1 (2.4)	2 (5.2)	1 (2.5)	0.5[a]	0.4[b]	4 (3.4)	[e]	[e]
Cancelled cycle between EPU and ET (%)	7 (17.5)	2 (4.9)	9 (23.1)	7 (17.5)	0.1[a]	0.5[b]	18 (15.8)	0.83 (0.32, 2.17)	0.6
Failed fertilisation	2 (5.0)	1 (2.4)	2 (5.1)	2 (5.0)	0.9[a]	0.9[b]	4 (4.2)	1.21 (0.23, 6.50)	0.8
Insufficient embryos	2 (5.0)	0 (0.0)	0 (0.0)	2 (5.0)	0.3[a]	1.0[b]	2 (1.7)	0.32 (0.04, 2.37)	0.3
OHSS	1 (2.5)	0 (0.0)	2 (5.1)	0 (0.0)	0.2[a]	1.0[b]	2 (1.7)	0.66 (0.06, 7.49)	0.7
Freeze all	2 (5.0)	1 (2.4)	5 (12.8)	3 (7.5)	0.3[a]	0.3[b]	9 (7.5)	1.54 (0.32, 7.45)	0.6
TOTAL CANCELLED	13 (32.5)	14 (34.1)	19 (48.7)	13 (32.5)	0.4[a]	0.7[b]	46 (38.3)	1.29 (0.61, 2.75)	0.5

[a]Chi-square; [b]Chi-square for trend across treatment groups; [c]Chi-square, comparison between placebo and any melatonin [d]Includes 'premature LH surge' and 'medication errors'; [e]insufficient sample to create a meaningful comparison statistic

In addition, the clinical pregnancy rate (per cycle started) in the placebo group was relatively low (15%) comparative to the literature, but the confidence interval around this point estimate is wide (95% CI 5.7%, 29.8%), suggesting that the true placebo pregnancy rate in this study could be as high as 29%. In order to ensure that this relatively low success rate was not a reflection of small sample size, I assessed the intention-to-treat clinical pregnancy outcomes of women who had declined participation in the trial (who were therefore, were eligible for inclusion and comparable to the placebo group). In this population of 211 women, the clinical pregnancy rate was 16% per cycle started. Sixty-four percent reached embryo transfer (compared with 63% in MIART placebo group) and 12% had a cancelled cycle before oocyte retrieval (compared with 15% in MIART placebo group). Reassuringly, these results are comparable to the MIART placebo group.

Despite its pilot nature, the MIART trial has several defining qualities that help to make it a high-quality addition to the literature on this subject. It is the first melatonin trial in IVF patients that is both randomised and placebo-controlled. It also investigates melatonin in isolation (without combination with other adjuvant therapies) and using a placebo-control as opposed to a within-subjects design where patients are used as their own controls. Together with being double-blinded, this helps to minimise bias and to ensure that results are as transparent as possible. The study was not funded by interested parties and therefore, reporting bias is also minimised.

Finally, the choice to investigate several doses of melatonin has proven worthwhile, as I have been able to show that there is a dose-dependent change in both serum and follicular fluid concentrations of melatonin. This suggests that oral administration of melatonin restricted to the ovarian stimulation period is sufficient to achieve supraphysiological concentrations of melatonin in both serum and follicular fluid. It has also demonstrated that low doses previously investigated may not have been sufficient to achieve biochemical changes in

melatonin concentrations in target tissues. This, together with the small sample sizes in former studies, may offer an explanation for previously negative findings, and suggests that any further efforts should concentrate on higher doses.

6.3 CLOSING REMARKS AND FUTURE DIRECTIONS

In order to definitively determine whether or not melatonin can improve IVF success rates, the design of a large multicentre double blinded randomised placebo-controlled trial is required. Because I have shown that lower doses of melatonin (2mg BD) do not achieve an increase in serum or follicular fluid concentrations of melatonin, an ideal dose would be closer to 8mg BD. I feel that it is unlikely that the lower dose would show a clinical difference, which is true of previously published data.

In addition, I have not demonstrated an increase in adverse events with high doses, and have shown a lack of effect on sleep and daytime sleepiness even with twice daily dosing. This safety data should assist with counselling and recruitment of participants to a larger trial. Assuming a withdrawal rate of 6% (similar to what was experienced in the MIART trial), to detect a difference in clinical pregnancy rate of 6.7% (from 15.0% in the control group to 21.7% in the treatment group) such a trial would require almost 1500 patients to be recruited (1334 analysed at 80% power, α=0.05, 1:1 allocation ratio). It should also be noted that MIART found a non-statistically significant difference in cancelled cycle rate of 19.3% in the any melatonin group compared with 5.6% in the placebo group (per protocol) ($p<0.07$). Therefore, in the design of a future trial, it would be prudent to arrange an interim analysis for safety following recruitment of 180 patients (90 to each intervention, power 80%, α=0.05). If no significant difference in cancelled cycle rates or other adverse effects is confirmed at this stage, then the trial should be allowed to continue to completion.

Using one IVF clinic, and the recruitment methods employed in MIART, this would take an estimated 20 years of recruitment. Therefore, to be feasible over a practical time-frame, this would require the involvement and cooperation of multiple IVF clinics.

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Appendices

Note: Appendices have been formatted from their original form to conform to this thesis

Appendix A: Standard Operating Procedures for the MIART Trial

Introduction

High levels of oxidative stress can have considerable impact on the outcomes of in-vitro fertilization (IVF). Recent studies have reported that melatonin, a neurohormone secreted from the pineal gland in response to darkness, has significant anti-oxidative capabilities which may protect against the oxidative stress of infertility treatment on gametes and embryos. Early studies of oral melatonin (3-4mg/day) in IVF have suggested favourable outcomes. However, most trials were poorly designed and none have addressed the optimum dose of melatonin. We present a proposal for a pilot double-blind randomised placebo-controlled dose-response trial aimed to determine whether oral melatonin supplementation during ovarian stimulation can improve the outcomes of assisted reproductive technology.

Aims

The overall aim of this trial is to determine whether oral melatonin administration can improve the outcomes of IVF/ICSI. In order to address this, we will determine whether melatonin administration has a dose-response effect in women undergoing IVF/ICSI on:

Biochemical markers of follicle and oocyte health (melatonin, 8-hydroxy- 2'-deoxyguanosine (8-OHdg), progesterone, oestradiol)

Sonographic markers of follicle health

Patient sleepiness

Pregnancy rate following IVF/ICSI

Methods and analyses

We will recruit 160 infertile women into one of four groups: placebo (n=40); melatonin 2mg twice per day (n=40); melatonin 4mg twice per day (n=40) and melatonin 8mg twice per day (n=40). The primary outcome will be clinical pregnancy rate. Secondary clinical outcomes include oocyte number/quality, embryo number/quality and fertilization rate. We will also measure serum melatonin and the oxidative stress marker, 8-hydroxy- 2'-deoxyguanosine (8-OHdg) at baseline and after treatment and levels of these in follicular fluid at egg pick-up. We will investigate follicular blood flow with Doppler ultrasound, patient sleepiness scores, and pregnancy complications, comparing outcomes between groups. NB: For funding reasons, blood tests and follicular fluid samples will only be taken from 25 patients per group (100 patients in total). Follicular flow 3D ultrasound will be used in 60 patients (total of 15 per group). Measurements of uterine artery blood flow will also be taken from these patients.

This Standard Operating Procedure describes the procedures required to undertake this study in a standard manner.

Preparation before recruitment commences

Transfer of medication to pharmacy

After compounding of melatonin and placebo tablets, Shavi Fernando (SF) will transfer the tablets from the compounding pharmacy to the Monash Health Pharmacy.

Randomisation of medication

JM will randomly allocate each trial group a letter from A to D using opaque envelopes eg. A = Melatonin 2mg bd and notify the pharmacy as to which doses should be allocated which letter. The Monash Health pharmacy will then remove current labels on each bottle and re-label each bottle with a letter (A to D) corresponding with the appropriate dose. Both JM and the Monash Health Pharmacy will keep a record of which letter represents which dose. No other member of the research team (including SF) will know this allocation until after analysis of data.

Storage of medication

These letter-labeled bottles will then be stored in the pharmacy at under 25 degrees celsius. Patients will be asked to store medication bottles in the refrigerator under 25 degrees celsius.

Safety of medication

The Data Safety and Monitoring Committee will be provided with an interim analysis of adverse events after 50% of participants have been enrolled.

Process of Recruitment

Specialist for review of results-Activation of treatment

The specialist will be sent the trial information, including PICF, ethics approval letter, medical defence letters to send to their MDO and an advertising poster to hang in their waiting room.

Pt attends for visit and the specialist will decide that the patient requires IVF.

IVF clinicians will offer patient study information pack (Plastic pocket with PICF in it with a sticker on front with main incl/excl criteria for doctor to quickly check before handing out). The clinician will provide the pack to the patient and stick the patients ID sticker onto the 'details page'.

Process of Notification

At the end of the consulting day, the specialist will give these page(s) to their secretary who will send a copy together with a copy of each patient's most recent pelvic ultrasound via email to SF to notify him of the patient's contact details.

Once receiving this call, SF will update the CONSORT table for the patient as 'approached'.

Equipment

Patient introductory pack - Introductory letter, envelope with PICF inside, SF's email address

Clinician pack - Introductory letter and trial summary, page with full inclusion/exclusion criteria, poster for clinicians' rooms, PICF, ethics approval letters, medical defence letter, patient details forms

Before Nurse visit

Pre-visit routine IVF tests will be followed up by SF with the external lab/Specialist that performed the tests.

SF will perform a followup call to the patient approximately 1-2 days after patient receives the information package, to determine the patient's interest and if they have any further questions.

If the patient is still interested in inclusion and eligible, SF will also ask the patient when they anticipate commencing IVF, when day 1 of that cycle will be and who their nurse is and that he will meet the patient after their nurse visit.

SF will then contact Deborah Deguingand (DD) or the nurse directly, who will book the patients nurse visit with an extra 30-60 minutes and tell SF the date and time of the appointment.

Prior to the nurse visit, SF will collect some bottles from each group (lettered) bottle from Monash Health pharmacy and take it to Monash IVF to give to the appropriate patient

Equipment

Patient record sheet

Consent form (PICF)

Trial Medication

Labels for medication (Trial specific ID and letter reference for medication)

Fridge at less than 25 degrees C

First visit (alongside nurse visit)

SF will have a list of potential participants attending the clinic on that day (previously prepared - see above). He will bring medication bottles from each treatment group and ActiWatches with him to dispense to the according patients (and blood collection tubes)

Routine nurse visit occurs

Immediately following nurse visit, SF will meet the patient and talk to them in the interview room (Suite 1) or research room (Suite 3, MSPH)

If at this stage she decides not to participate, the medication will be returned to the Monash Health Pharmacy. Her CONSORT table will be updated to reflect 'not recruited'.

If she agrees to participate, SF will:

Obtain written informed consent on the PICF and provide the patient with the appropriate trial medication.
Ask the patient to note any medication she is currently taking including complementary therapies, and instruct her to stop taking complementary therapies (including acupunture) from this point until the embryo transfer
Add this information to Patient Record Access Database.
Measure and record the patient's age, BMI, smoking status and parity and complete the remainder of the 'patient details' on the Patient Record.
SF will randomise the patient accordingly:

Randomisation procedure:
SF will enter the details into the minimization program and identify which letter (A-D) trial medication to provide to the patient and provide it to the patient

Instructions for patient after randomisation
Inform them to bring in the bottle at the embryo transfer for return for capsule counting
Provide them with an ActiWatch together with instructions and ask her to begin wearing it
The patient will be provided with a unique trial identifier number in sequence with enrollment and this will be entered onto her Patient Record. She will also be asked to complete the validated sleep diary.
SF or DD will take 8.5ml whole blood in each of 3 BD serum tube for baseline levels of melatonin and 8-OHdg.
After the patient has left the consultation:
Update the CONSORT table to include the patient as being 'recruited'
Notify the nurse of trial inclusion and nurse will place a 'MELATONIN TRIAL' sticker on graph. This sticker will alert the nurse that the patient is on the melatonin trial and is to be scanned (for follicular Doppler flow also) on Day 8-9 by Nikki White (NW) on Tues, Wed or Thurs at 0700 (and, less preferably on Mon or Fri at 0700)

Post randomization- transport of blood samples
The tube will be shielded in a light-proof container and labelled with a patient ID number and the letter 'a' to signify that this is the first sample of the protocol and kept on ice until transferred to the lab.
SF will take the blood samples back to MIMR

Post randomization labeling and storage of blood samples
At MIMR or Hawthorn blood samples will be centrifuged, pooled and then aliquoted into 5 eppendorf tubes (1.5ml per 2ml eppendorf tube), labeled with the patient trial ID, the date, 'serum', letter 'a$' (to represent that this is the first serum sample) and 'SF/EW' and frozen at -80C. Any remaining serum

will be aliquoted into upto another 2 eppendorf tubes and labelled 'a$', 'serum' and 'e' for 'extra' and 'SF/EW'. Refer to Appendix A. If insufficient blood is collected, freeze in aliquots of 1.2ml in a minimum of 2x 2ml eppendorf tubes.
SF will then bring the patient's trial prescription to Monash Pharmacy so that a record of which patient received which medication can be kept.

Equipment
Patient record sheet
Consent form (PICF)
ActiWatch and sleep diary
Medication bottle
Patient diary form and Sleepiness scale
Appropriately allocated Trial Medication
3x BD serum tubes - labelled with date, patient ID, 'SF/EW' and 'a$' (eg. 1-9-14, SF/EW, 23a$)
Freezer at -80C
5x Labeled eppendorf tubes (plus any extra labeled tubes)

Day 2 of period-Day 1 of stimulation
At the commencement of FSH injections, the patient also commences her trial drug orally, twice per day at 1000 and 2200.
They will need to take tablets at least 12 hours apart
They will need to record the exact time of taking each tablet on the diary form.
If a tablet is missed, they should take it when it is remembered and record the time on the diary form.
Equipment
Trial drug (patient should already have this in her possession)

Day 8-9-Scan day
At the time of her ultrasound, measurements will also be taken for the ovarian follicular blood flow from each ovary (only 15 patients per group - see note above). Measurements of uterine artery blood flow will also be taken.
The patient will be contacted via telephone (See 'Day 8 phone interview checklist') to determine whether any adverse events have been experienced, and these will be recorded on the adverse events form. At the same time she will be reminded to bring medication bottle, Actiwatch and completed diaries to the oocyte retrieval.
Equipment
Ultrasound and sonographer - follicular flow and uterine artery flow
Adverse events form

Prior to EPU- Instructions for patient

SF will provide Instructions for nurses in relation to the trial as the nurses will be ringing them to tell them to take trigger injection or give the patient the EPU transfer time

The night before EPU, the participant will cease her trial medication,

The night before EPU the participant will continue to wear her Actiwatch

Prior to EPU-Investigator task

The night before EPU, SF will ensure that a tube of 5ml medium has been set aside to allow it to be equilibrated in the standard medium warmer for use the following day

Day of EPU-patient

On the morning of EPU, SF will check to see if the patient has completed her sleepiness scale and daily diary

SF will enquire as to whether the subject has experienced any adverse events

The patient will return the medication bottle to SF who will count and document remaining tablets to ascertain compliance on the separate Drug Compliance Chart. The patient will return the Actiwatch and both diaries

Day of EPU-Investigator

SF or DD will be present for the EPU. At the time of inserting the IVC for anaesthesia, 3 x BD serum tube will be taken for melatonin and 8-OHdg with 8.5ml whole blood each.

The tubes will be shielded in a light-proof container and labeled with a patient ID number and the letter 'b$' to signify that this is the second serum sample of the protocol.

At the time of oocyte collection, the clinician will obtain a clean specimen of follicular fluid from the first and largest accessible follicle in each ovary according to the below procedure (Luck, Jeyaseelan et al. 1995). It should be noted that this does not necessarily represent the 'largest follicle' as if the largest follicle was not immediately accessible, the most accessible follicle was aspirated.

Procedure:

SF will prepare four collection tubes with exactly 2ml of medium in each (2 x L and 2 x R)

Nurse will prime the aspiration line into a follicular fluid tube which will then be set aside (FT1). The previously filled tube (L) will then be used for the follicular fluid sample from the largest easily accessible follicle from the left ovary. If the follicle is large, the second L tube will be used for overflow. Before flushing this follicle, the clinician will hand the filled tube to SF, then continue with the aspiration as normal from the left ovary into different tubes (can then use FT1).

Nurse will then flush the aspiration line with into another follicular fluid tube (FT2) between ovaries. The previously filled tube (R) will then be used for the follicular fluid sample from the largest easily accessible follicle from the right ovary. If the follicle is large the second R tube will be used for overflow Before flushing this follicle, the clinician will hand the filled tube to SF, then continue with the aspiration as normal from the right ovary into different tubes (can then use FT2).

The L and R tubes will be handed to the embryologist for oocyte extraction

L fluid (after the oocyte is removed) will be given to SF and pipetted into a shielded BD Falcon tube and the volume noted

R fluid (after the oocyte is removed) will be given to SF and pipetted into a shielded BD Falcon tube and the volume noted

The follicular fluid from L and R will be individually separated and stored.

Transport of samples post EPU

Follicular fluid

SF will take labeled L and R and the labeled serum BD tubes in an Esky on ice to MIMR for processing

SF will note (and subjectively score: none, mild, mod, severe) whether FF is contaminated with blood and whether the serum is lipaemic. This is performed by visual inspection and subjective assessment of the sample

SF will spin L down to separate supernatant (follicular fluid) and granulosa cells,

SF will aliquot the follicular fluid into 3 eppendorf tubes (1.5ml per 2ml eppendorf tube), labeled with date, patient ID and 'L' and 'SF/EW'. Remaining follicular fluid from the L sample will be stored in 2x eppendorf tubes labelled L and 'e' for 'extra' (depending on volume of FF) with date, ID and 'SF/EW' and 'FF'.

This process will be repeated for R: SF will spin R down to separate supernatant (follicular fluid) and granulosa cells, aliquot the follicular fluid into 3 eppendorf tubes (1.5ml per 2ml eppendorf tube), labeled with date, patient ID and 'R' and 'SF/EW' and 'FF'. Remaining follicular fluid from the R sample will be stored in 2x eppendorf tubes labelled R and 'e' for 'extra' (depending on volume of FF) with date, patient ID and 'SF/EW'.

Granulosa Cells

Perform procedure separately for each collection tube ie. left and right (but can be performed concurrently).

After spinning and removing follicular fluid, a pellet of cells should remain in the base of the BD collection tube.

Warm red cell lysis buffer in waterbath to 37C

Add 1ml of red cell lysis buffer to BD tubes (left and right), and resuspend the cells until red cells are completely lysed - add more red cell lysis buffer if required as quickly as possible

Dip in waterbath for a total of 1 minute (both tubes).

Add pink media up to total volume of 10ml per tube (left and right)

Spin samples at 1200g for 5 minutes at 21C

Prepare 2 wells in trypan blue plate with 10uL of trypan blue in each (one for left and one for right samples)

Prepare and clean the haemocytometer

Once samples have finished being centrifuged, use glass pipette attached to wall suction to remove as much excess medium as possible

Resuspend in 1ml pink medium in each of the two blue top BD tubes (left and right)

Pipette 10uL of this and resuspend this into the well of trypan blue (do this for both the left and right collection tubes) - (should have a total of 20uL volume (10uL trypan blue and 10uL cell medium) in each well by now)

Pipette 10uL of each well into cell counter top for right and bottom for left

Do cell count

Calculate cell count using:

Total cells = (cell count/4) x 10^4 x 2 x 1

ie. Total cells = (cell count/number of squares) x 10^4 x dilution factor x volume

Record this count in lab book.

Label 2x1.5ml eppendorf tubes with patient ID, date, 'GC' and 'L' for left or 'R' for right and 'SF/EW'(1xeppendorf for each follicle sample)

Resuspend the pellet

Transfer the cells/media into 2ml eppendorf tube (left and right)

Centrifuge again at 1200g for 5 mins at 21C

Remove as much media as possible

Add 200uL RNA later (each to left and right)

Freeze at -80C in the 'Granulosa cell' eppendorf box in Rack 16 at the back.

Blood

SF will shield and take the blood samples taken prior to anaesthesia back to MIMR, where they will be centrifuged, aliquoted into 5 eppendorf tubes, labelled with the patient ID, date, 'SF/EW' and the letter 'b$' (second serum sample). Any remaining serum will be aliquoted into up to another 2 eppendorf tubes (depending on volume of remaining serum) and labeled with patient ID, date, 'serum', 'SF/EW', 'b' and 'e' ('extra').

Equipment

Patient Record

Drug Compliance Chart

Return of medication bottle

Return of ActiWatch and sleep diary

Patient sleepiness scale to be returned

Follicular fluid collection tubes preprepared with 2ml medium each x 4 (L, R)

Serum BD tube x 3 - labeled with date patient ID and 'b$' (eg. 23b$)

labeled follicular fluid tube with date, 'SF/EW', trial ID and 'L' (eg. 23L)

labeled follicular fluid tube with date, 'SF/EW', trial ID and and 'R' (eg. 23R)

11 x Eppendorf tubes labeled with ink (3 for left and 3 for right follicle, 5 for serum)
Granulosa cell storage, labelling and transport

Post EPU

SF will return the used bottle to Monash Health Pharmacy who will store it in case of need for future reference.
SF will record compliance in the Patient Record Access Database
Results from routine blood tests taken for oestradiol, progesterone and LH will be obtained from external pathology centre and recorded in Patient Record Access Database

Embryo transfer occurs or does not occur

HCG at D16 if Embryo transferred

Blood is taken for HCG and progesterone routinely and results will be followed up with the external lab performing the tests.
SF will follow up the HCG result electronically

Positive HCG

SF will follow up HCG result every week (with the external pathology lab performing the tests) until either miscarriage (this will then be recorded including the gestation at diagnosis and absolute HCG levels) or 7/40 US.
The results of the 7/40 US will then also be recorded, the due date for the pregnancy identified
20/40 US followed up and any fetal abnormalities recorded and the pregnancy will be followed up to assess the outcome of live birth.
NB: (*Only after the live birth, will the patient, investigators and clinicians be allowed to be unblinded.*

Negative HCG

If the initial HCG level (or any subsequent one) is indicative of pregnancy failure, management will depend on the patient's wishes. When she sees her specialist at her next visit (usually 2-4 weeks after confirmed failed transfer) her specialist will offer her the option of continuing on the trial or withdrawing from the trial.
If she wishes to continue with a further cycle on the trial, she will be randomised to *one of the other* treatment arms. In this way, she will be guaranteed at least one active arm over 2 cycles.
The trial protocol will then proceed, with particular attention being paid to sleep disturbances and adverse events.

Equipment

Access to results from pathology and ultrasound services

Privacy and confidentiality of data leaving Monash IVF

All samples that leave MSPH will be identified only by a unique 'trial ID' which will be allocated to the patient at the time of randomisation

At the end of collection, there should be the following blood samples for a total of 100 patients (25 per group):

Progesterone (3 time points)

Oestradiol (3 time points)

LH (one time point)

Melatonin (two time points)

8-OHdg (two time points)

At the end of collection, there should be the following follicular fluid samples for each of a total of 100 patients:

Melatonin (1 sample)

8-OHdg (1 sample)

Second cycle

The second stimulation cycle with trial medication will not require an Actiwatch. It will only require the alertness score, partial sleep diary and the compliance diary. Similarly, the baseline blood test will not be required. Only the blood test at oocyte collection and follicular fluid and GCs will be collected.

Embryos frozen from first (trial) cycle

The second cycle in this case will occur as per usual (non-trial) care. That is, a patient will not be given melatonin if frozen embryos obtained from a melatonin stimulation cycle are to be used.

After their first negative HCG, call patient and advise that if they require a further stimulation cycle to obtain more oocytes, that they will be able to continue a further trial cycle with a different trial medication

There is no obligation to continue the trial

No frozen embryos from first (trial) cycle

Notify SF and DD. Offer patient a different trial medication (in sequence) in their second cycle, and to mention this to their specialist if they wish to continue the trial

There is no obligation to continue the trial

Special Circumstances

If cycle is cancelled before oocyte collection

This may occur for example, if insufficient follicles are developed during the stimulation cycle, and despite repeat ultrasounds and giving more time, follicles don't develop to an acceptable level before oocyte retrieval or in the case of OHSS.

If this occurs: Notify SF and DD and mark the patient as 'cancelled before EPU' on the database

Make a note on the database as to why cycle was cancelled in the 'notes' section

Record Day 8 ultrasound results

Record Day 8 blood tests

Contact the patient, offer sympathies and offer inclusion in the trial for the following cycle with a different trial medication

If cycle is cancelled before embryo transfer

This may occur for example, in the case of OHSS.

If this occurs: Notify SF and DD, Contact the patient and explain that once the embryo is transferred, the results of the transfer will be followed up as per trial protocol

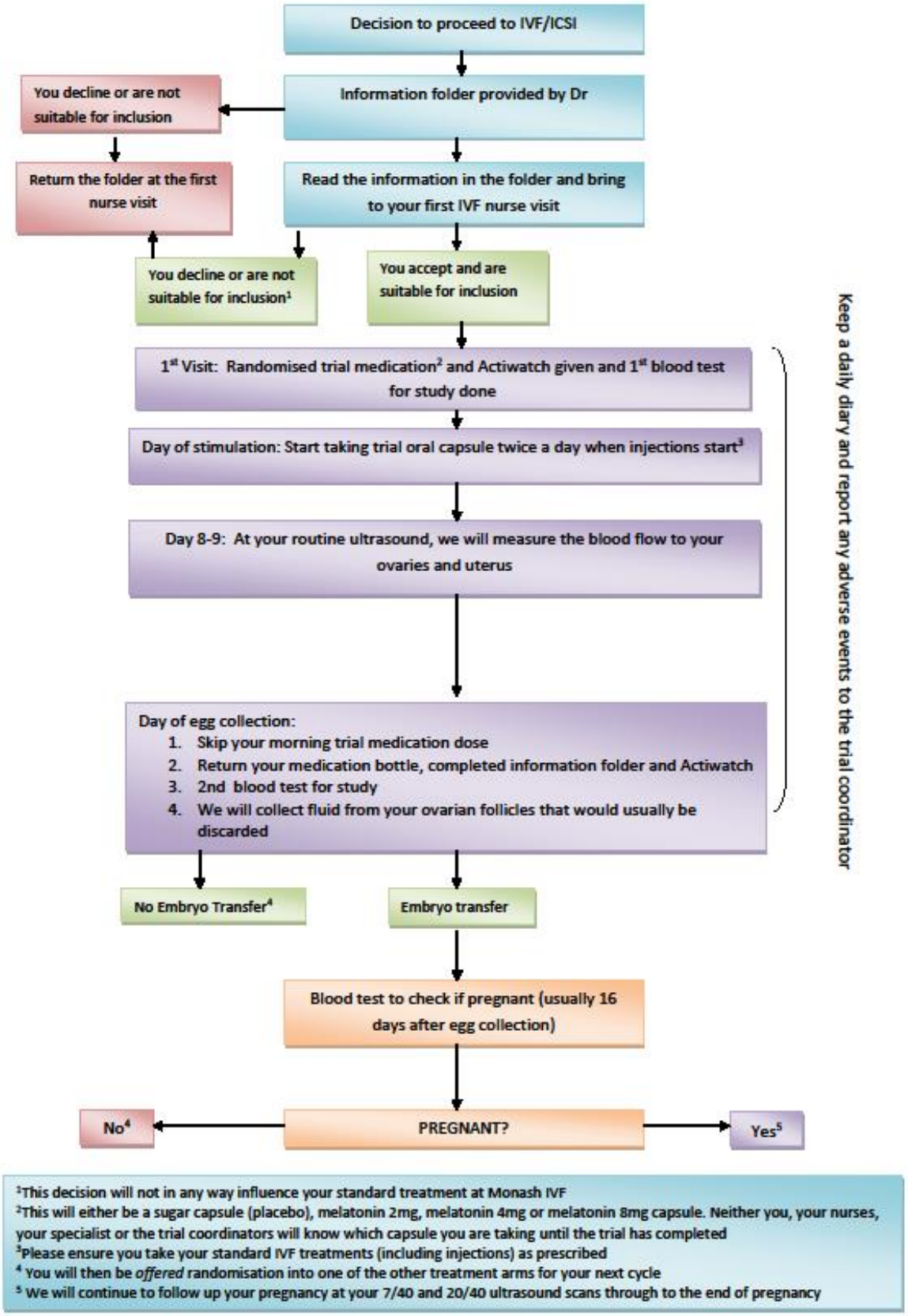
Record day 8 US and blood test results, number of embryos and all other trial data possible

Mark patient as 'cycle cancelled before embryo transfer' on database

Once embryo is transferred, mark this in appropriate section on database

Follow up outcome of embryo transfer

Appendix B: Patient flowchart



Appendix C: Patient information and consent form

Monash Surgical Private Hospital - Monash IVF

Participant Information Sheet/Consent Form

Interventional Study - *Adult providing own consent*

Monash Surgical Private Hospital

Title	**Melatonin and infertility: Can we improve outcomes of assisted reproductive technology – a placebo-controlled randomised controlled trial**
Short Title	**Melatonin and infertility**
Coordinating Principal Investigator/ Principal Investigator	*Prof Luk Rombauts* *Dr Shavi Fernando*
Associate Investigator(s)	*Prof Euan Wallace* *Dr Tiki Osianlis* *A/Prof Beverley Vollenhoven*
Associate Researcher(s)	*Ms Caroline Motteram*
Location	*Monash IVF*

Part 1 What does my participation involve?

1 Introduction

This research project is being undertaken at Monash Health and Monash IVF.

You are invited to take part in this research project. This is because you have had difficulty conceiving naturally and have been advised to undergo invitro fertilisation (IVF). The research project is testing a new complementary treatment for infertility. The new treatment is called melatonin.

This Participant Information Sheet/Consent Form explains the research project. It describes the tests and treatments involved. Knowing what is involved will help you decide if you want to take part in the research.

Please read this information carefully. Ask questions about anything that you don't understand or want to know more about. Before deciding whether or not to take part, you might want to talk about it with a relative, friend or your local doctor.

Participation in this research is voluntary. If you don't wish to take part, you don't have to. You will continue to receive the most appropriate care whether or not you take part.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it you are telling us that you:

• Understand what you have read

• Consent to take part in the research project

• Consent to have the tests and treatments that are described

• Consent to the use of your personal and health information as described.

You will be given a copy of this Participant Information and Consent Form to keep.

2 What is the purpose of this research?

You have been diagnosed with infertility and your doctor has recommended treatment with a form of assisted reproductive technology (ART) or which in-vitro fertilisation (IVF) is a type.

It has been established that when undergoing ART, eggs and embryos may interact negatively with oxygen molecules in a process called 'oxidative stress'. This oxidative stress has the potential to damage eggs and embryos.

Melatonin is an antioxidant and may be able to help reduce the effect of 'oxidative stress' on eggs and embryos.

Our research project would like to determine whether the use of oral melatonin can improve success rates of ART.

The main aims of this trial are:

a) To determine the effect of oral melatonin supplementation on fertilisation, clinical pregnancy, and live birth rates and to determine an optimal dose if such a relationship exists.

b) To determine the effect of oral melatonin supplementation on biochemical parameters and body fluid levels of melatonin and thereby to determine its effect on oxidative stress in the body.

We hope to show that melatonin can reduce the negative effect of oxidative stress on embryos and therefore, increase success rates of ART. If this study is successful, we would hope that melatonin would become a standard complementary therapy for all patients undergoing ART for infertility.

It is necessary for us to undertake this trial because others have performed similar trials with varying degrees of uncertainty. Previous trials have not succeeded in proving or disproving the beneficial effects of melatonin. Because of this, doctors are not certain whether melatonin has a beneficial effect or not. Trials that have been completed to date have shown the safety of oral melatonin, but none have addressed specifics regarding dose and none have actually proven a benefit in participants with infertility.

This trial seeks to fill these gaps in knowledge by performing a large trial testing different doses of oral melatonin. This trial, once completed, should add to our knowledge about melatonin and its effect on infertility, and should help to confirm (or refute) the benefit of melatonin in infertility.

Melatonin is approved in Australia to treat primary insomnia under the trade name, Circadin®. However it is not approved to treat infertility. Therefore, it is an experimental treatment for infertility. This means that it must be tested to see if it is an effective treatment for infertility.

The results of this research will be used by the study doctor, Dr Shavi Fernando to obtain a *Doctor of Philosophy postgraduate* degree (PhD).

This research has been initiated by the study doctor, Dr Shavi Fernando.

This research has been funded by the Ritchie Centre.

This research is being conducted by The Ritchie Centre in collaboration with Monash IVF.

3 What does participation in this research involve?

This research project will run for a total of three years.

Participation in this research means that you will be in regular touch with the research team from the moment you enrol until the birth of your baby or withdrawal from the trial.

You have been asked to participate in this trial because you meet the inclusion criteria:

- You have not been able to conceive naturally after 12 months of regular intercourse without use of contraception
- You are between 18 and 45 years of age
- You have a body mass index between 18 and 35
- You require in-vitro fertilisation (IVF) or intra-cytoplasmic sperm injection (ICSI)
- You are cycling regularly

Once you have consented to participate in the trial:

- You will be participating in a randomised controlled research project. Sometimes we do not know which treatment is best for treating a condition. To find out we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same, each participant is put into a group by chance (random).
- You will be participating in a double-blind study. This means that neither you nor your study doctor will know which treatment you are receiving. However, in certain circumstances your study doctor can find out which treatment you are receiving.
- You will be allocated to receive one of four study drug protocols:
    - Placebo twice per day (A placebo is a medication with no active ingredients or a procedure without any medical benefit. It looks like the real thing but is not)
    - 4mg melatonin (2mg tablet twice per day)
    - 8mg melatonin (4mg tablet twice per day)
    - 16mg melatonin (8mg tablet twice per day)
- Therefore, you have a 3 in 4 chance of receiving one of the active ingredient medications.
- You will also be asked to wear an 'Actiwatch' which is a small watch-like device used to monitor sleep patterns from the time of enrolment to the time of egg collection, and to complete a simple daily sleep diary and a summary sleepiness score.
- We will obtain only relevant information regarding your medical and fertility history, sometimes from your treating clinician, local doctor and/or Monash IVF with your clinician's permission

During melatonin treatment:

- You will take your study medication twice per day. It does not matter whether this is with or without food.
- You will be asked to donate a maximum of 60mL (4 tablespoons) of your own blood:
    - During the week before you start your stimulation cycle (30ml)
    - Day of your egg pick up (30ml)

    These tests will assess melatonin levels and levels of oxidative stress. At these visits, you are asked to report any problems or issues that have occurred to a member of the research team.
- At the time of your egg pickup, fluid that normally surrounds the egg is usually discarded. Instead of disposing of this fluid immediately, we will collect it to test the levels of melatonin and oxidative stress in the fluid surrounding the egg.
- You will continue melatonin treatment until egg pick up.
- After your embryo transfer:
    - If you become pregnant

        - Your due date will be recorded and a member of the research team will follow up the outcome of your ultrasounds and delivery once your baby is born
    - If you are not pregnant, you will be offered to continue with the trial for one further cycle. If this is the case, you will be offered a randomly chosen medication in this second cycle. You will be guaranteed at least one dose of melatonin over the two cycles. If this is the case, no break between cycles will be required as melatonin remains in the body for only a very short time.
    - If you are not pregnant and decide you want to complete future cycles *without* being part of the trial, this will be permitted.
    - If you are not pregnant and decide you *do not want further ART treatment*, you will be discharged from Monash IVF and the trial.

This research project has been designed to make sure the researchers interpret the results in a fair and appropriate way and avoids study doctors or participants jumping to conclusions

There are no additional costs associated with participating in this research project, nor will you be paid. All medication, tests and medical care required as part of the research project will be provided to you free of charge.

It is desirable that your local doctor be advised of your decision to participate in this research project. If you have a local doctor, we recommend that you inform them of your participation in this research project.

4 What do I have to do?

During the trial, there aren't any specific restrictions on physical activity or diet apart from what is recommended by your infertility doctor.

If you take regular medication that is necessary for the treatment of medical conditions, these should *not* be ceased. We do request, however, that you notify the research team of any medications you are continuing to take or begin taking during the study period.

We also ask that, apart from standard pregnancy vitamins (folate, pregnancy multivitamin etc) recommended by your fertility doctor, you do not take any other non prescribed complementary therapies. If you do, please inform the study coordinator.

You will also be asked to wear an 'Actiwatch' (see above) and complete a daily sleep diary.

While you are attempting to get pregnant, we recommend that you do not donate blood to a blood bank.

To be eligible for the trial you must be between 18 and 45 years old, have a body mass index between 18 and 35 and be having your first cycle of IVF/ICSI and have a Medicare number.

You will not be eligible to participate in this study if you have one or more of the following:

- □ Current **untreated** pelvic pathology – Stage 3 or 4 endometriosis, large submucosal uterine fibroids/polyps thought by the specialist to affect fertility, diagnosed current pelvic inflammatory disease, uterine malformations (i.e uterine didelphys, bicornuate uterus and septate uterus), Asherman's syndrome and the current diagnosis of a hydrosalpinx (not treated).
- □ Currently enrolled in another investigational trial
- □ Concurrent use of other adjuvant therapies (eg. Co Enzyme Q10, acupuncture)
- □ Current pregnancy
- □ Malignancy or other contraindication to IVF
- □ Autoimmune disorders
- □ Will not have regular blood tests with Monash IVF (because of distance from Monash IVF)
- □ Undergoing preimplantation genetic diagnosis (PGD)
- □ Hypersensitivity to melatonin or its metabolites

- Concurrent use of any of the following medications
    - Fluvoxamine (eg. Luvox, Movox, Voxam)
    - Cimetidine (eg. Magicul, Tagamet)
    - Quinolones and other CYP1A2 inhibitors (Ciprofloxacin, Avalox)
    - Carbemazepine (eg. Tegretol), rifampicin (eg.Rifadin) and other CYP1A2 inducers
    - Zolpidem (eg Stilnox), zopiclone (eg. Imovane) and other non-benzodiazepine hypnotics

If you agree to participate, we expect that you will continue to use the study medication in its entirety and as prescribed.

5 Other relevant information about the research project

- This trial will include a total of 160 patients (40 patients in each of the study groups) treated through the Monash IVF group involving collaboration with the Ritchie Centre at Monash Medical Centre.
- There are four study groups of varying doses of melatonin (as mentioned above) including a placebo group
- This is a crossover trial, and after your first cycle, you will be offered to try one of the other medications (chosen at random) for your second cycle.

6 Do I have to take part in this research project?

Participation in any research project is voluntary. If you do not wish to take part, you do not have to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

If you do decide to take part, you will be given this Participant Information and Consent Form to sign and you will be given a copy to keep.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with *Monash IVF or Monash Surgical Private Hospital.*

7 What are the alternatives to participation?

You do not have to take part in this research project to receive treatment at this hospital. Other options are available; these include obtaining your own melatonin from a pharmacy or using other complementary therapies together with melatonin at your own expense. As mentioned earlier, melatonin and many other adjunctive therapies have not yet been proven to work, and participation in this study will help determine the effectiveness of melatonin in this context. Your study doctor will discuss these options with you before you decide whether or not to take part in this research project. You can also discuss the options with your local doctor.

8 What are the possible benefits of taking part?

We cannot guarantee or promise that you will receive any benefits from this research. However, we believe that melatonin treatment will lower levels of oxidative stress in your body and improve the quality of your eggs and embryos. Oxidative stress seems to reduce the success rates of ART. The benefit from participating in this study is therefore the possible protection from oxidative stress and possible increase in pregnancy rates after ART. If Melatonin is shown to work, we may also determine the optimal dose and therefore allow for future patients to benefit from the outcome of this trial

9 What are the possible risks and disadvantages of taking part?

Melatonin

Melatonin has previously been used during infertility treatment, but only in small studies. These studies have not reported any problems in regards to these pregnancies or babies. You will only be taking melatonin from the commencement of your ovarian stimulation medication until your eggs are collected.

Patients should avoid alcohol before, during and after taking melatonin.

Melatonin has an excellent biosafety profile, but like any other medicine can have adverse reactions. As referenced by MIMS, these are considered to be uncommon side effects of melatonin (i.e., likely to occur in fewer than 1 in 100 patients):

- Melatonin can cause drowsiness, so you will need to be careful if you need to drive or use machines. If this concerns you, please discuss it with the research team.
- Irritability, nervousness, restlessness insomnia, abnormal dreams, anxiety, migraine, lethargy, psychomotor hyperactivity (restlessness associated with increased activity), dizziness, somnolence (tiredness), high blood pressure, (upper) abdominal pain, indigestion, mouth ulceration, dry mouth, hyperbilirubinaemia (changes in the composition of your blood which could cause yellowing of the skin or eyes (jaundice), inflammation of the skin (dermatitis, night sweats, pruritis (itching), rash, dry skin, pain in extremities, menopausal symptoms, asthenia (feeling of weakness), chest pain, excretion of glucose in urine, excess proteins in the urine, abnormal liver function and weight increase.

The following events are considered to be rare (i.e., likely to occur in fewer than 1 in 1,000 patients):

- Shingles, reduced number of white blood cells in the blood, decreased number of platelets in the blood, high level of fatty molecules in the blood, severe chest pain due to angina, feeling your heartbeat (palpitations). low serum calcium levels in the blood, low sodium levels in the blood, altered mood, aggression, agitation, crying, stress symptoms, disorientation, early morning awakening, increased sex drive, depressed mood, depression, loss of consciousness or fainting, memory impairment, disturbance in attention, dreamy state, restless legs syndrome, poor quality sleep, ‘pins and needles’ feeling (paresthesia) reduced visual acuity (visual impairment), blurred vision, watery eyes, dizziness when standing or sitting, vertigo, hot flushes, gastro-oesophageal reflux, gastrointestinal disorder, blistering in the mouth, tongue luceration, gastrointestinal upset, vomiting, abnormal bowel sounds, flatulence (wind), salivary hypersecretion (excess saliva production), halitosis (bad breath), abdominal discomfort, gastric disorder, inflammation of the stomach lining, eczema, erythema (skin rash), hand dermatitis, psoriasis, pruritic rash (itchy rash), nail disorder, arthritis, muscle spasms, neck pain, night cramps, increased duration of erection, inflammation of the prostate gland, tiredness, pain, thirst, passing large volumes or urine, presence of red blood cells in the urine, urination during the night, increased liver enzymes, abnormal blood electrolytes and abnormal laboratory tests.

If you are taking any drugs, please tell the member of the research team, because some types of medication should not be combined with melatonin. If you experience any of these effects, you should not drive, and should contact the study coordinator.

Data & Sample Collection

Your first blood test will be taken on the day of your nurse visit. This will be in addition to your routine care, and will be for the purposes of this research project only. As far as possible, we will try to collect your blood samples at the same time as blood is being collected for your normal medical care or when an intravenous drip is being placed.

Having a blood sample taken may cause some discomfort or bruising. Sometimes, the blood vessel may swell, or blood may clot in the blood vessel, or the spot from which tissue is taken could become inflamed. Rarely, there could be a minor infection or bleeding. If this happens, it is easily treated.

Counselling

If you become upset or distressed as a result of your participation in the research, the researcher is able to arrange for counselling or other appropriate support. Any counselling or support will be provided by staff who are not members of the research team. In addition, you may prefer to suspend or end your participation in the research if distress occurs. If you decide to suspend or end your participation in this research project, your care will not be affected, but we do ask that you notify us as to the reason of your withdrawal.

As with any research project, there may be additional risks that the researchers do not expect or do not know about.

Injury

If you suffer any injuries or complications as a result of this research project, you should contact the study team as soon as possible and you will be assisted with arranging appropriate medical treatment. If you are eligible for Medicare, you can receive any medical treatment required to treat the injury or complication, free of charge, as a public patient in any Australian public hospital. If you are not eligible for Medicare, please notify the trial coordinator, as you will not be eligible to participate in this trial.

10 What will happen to my test samples?

To protect your identity, once the tissue samples and information from your medical records have been collected, any personal information that could identify you will be removed from them e.g. name, hospital number, address, date of birth. The de-identified samples and information will then be allocated a unique code. The code assists the researchers who will be analyzing and interpreting the results of the research project, to relate your pregnancy information to the tissue samples without knowing who you are.

The samples collected for this research project:

- **Will** be analysed to assess markers of oxidative stress and melatonin levels only
- **Will** be used for the purposes of this research project only. However, the results from this project will be used by the researchers to inform future research projects.
- **Will not** be used to diagnose any conditions or guide your clinical care.
- **Will not** be used for genetic testing
- **Will not** be used for stem cell research.

Any information obtained in connection with this research project will remain confidential. Information will only be disclosed as required by law. Your health records and any information obtained during the study are subject to inspection (for the purpose of verifying the procedures and data) by the relevant authorities, Monash Surgical Private Hospital or as required by law.

By signing the attached Consent Form, you authorise release of, and access to, this confidential information by the relevant study personnel and regulatory authorities as noted above.

Written information will be securely stored in a locked filing cabinet and password protected database, accessible only by the named researchers. De-identified tissue samples will be stored in a laboratory in The Ritchie Centre, Monash Institute of Medical Research (MIMR) or Monash Surgical Private Hospital.

After the study is completed, the information and remaining tissue samples will be securely stored for 15 years by the principal investigator, as currently recommended by the National Health and Medical Research Council (NHMRC). After this time, all the information and tissue samples will be disposed of in a secure and confidential manner.

If you give us your permission by signing the Consent Form, it is intended that the project outcomes will be submitted for publication in peer reviewed medical journals and presented at meetings. In any publication or presentation, information will be provided in such a way that you cannot be identified in any way.

Your consent is requested to use your data collected for this study and in future research that is approved by the Monash Surgical Private Hospital Human Research Ethics Committee. You do not have to consent for both of these uses and you can specify which.

11 What if new information arises during this research project?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your study doctor will tell you about it and discuss with you whether you want to continue in the research project. If you decide to withdraw, your study doctor will make arrangements for your regular health care to continue. If you decide to continue in the research project you will be asked to sign an updated consent form.

Also, on receiving new information, your study doctor might consider it to be in your best interests to withdraw you from the research project. If this happens, he/ she will explain the reasons and arrange for your regular health care to continue.

12 Can I have other treatments during this research project?

Whilst you are participating in this research project, you may not be able to take some or all of the medications or treatments you have been taking for your condition or for other reasons. It is important to tell your study doctor and the study staff about any treatments or medications you may be taking, including over-the-counter medications, vitamins or herbal remedies, acupuncture or other alternative treatments. You should also tell your study doctor about any changes to these during your participation in the research project. Your study doctor should also explain to you which treatments or medications need to be stopped for the time you are involved in the research project.

13 What if I withdraw from this research project?

If you decide to withdraw from the project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to discuss any health risks or special requirements linked to withdrawing.

If you do withdraw your consent during the research project, the study doctor and relevant study staff will not collect additional personal information from you, although personal information already collected will be retained to ensure that the results of the research project can be measured properly and to comply with law. You should be aware that data collected by the sponsor up to the time you withdraw will form part of the research project results. If you do not want them to do this, you must tell them *before* you join the research project.

14 Could this research project be stopped unexpectedly?

This project could be stopped if we observe that maternal well being is being harmed by this treatment. This event seems to be very unlikely, but we will not take any risks and will discontinue this trial if there are too many participants showing possible adverse events.

15 Further information or concerns:

If you require further information or if you have any problems concerning this project, you can contact:
Dr. Shavi Fernando (first contact)
Telephone: [redacted] Email: [redacted]
Prof. Euan Wallace
Telephone: [redacted] Email: [redacted]

16 Who has reviewed the research project?

The ethical aspects of this research project have been approved by the Human Research Ethics Committee of Monash Health, Monash University and Monash Surgical Private Hospital. This project will be carried out accordance with the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about being a research participant in general, then you may contact:

Monash Health HREC, Research Support Services; T: [redacted]

Consent Form

Title	**Melatonin and infertility: Can we improve outcomes of assisted reproductive technology – a placebo-controlled randomised controlled trial**
Short Title	**Melatonin and infertility**
Coordinating Principal Investigator/	*Prof Luk Rombauts*
Principal Investigator	
Associate Investigator(s)	*Dr Shavi Fernando* *Prof Euan Wallace* *Dr Tiki Osianlis* *A/Prof Beverley Vollenhoven* *Caroline Motteram*
Location	*Monash IVF*

Declaration by Participant

I have read, or had read to me in a language I understand, this document. I understand the purposes, procedures and risks of this research project as described within it.
I have had an opportunity to ask questions and I am satisfied with the answers I have received.
I freely agree to participate in this project according to the conditions in the Participant Information.
I will be given a copy of the Participant Information and Consent Form to keep.
I understand that the researcher has agreed not to reveal my identity and personal details if information about this project is published or presented in any public form.
I consent to the data collected in this study being used in future related research that has obtained approval from the Monash Surgical Private Hospital Human Research Ethics Committee ☐ (only valid if ticked).

Name of Participant (please print) _____

Signature _____ Date _____

Name of Witness* to Participant's Signature (please print) _____

Signature _____ Date _____

* Witness is not to be the investigator, a member of the study team or their delegate. In the event that an interpreter is used, the interpreter may not act as a witness to the consent process. Witness must be 18 years or older.

Declaration by Study Doctor/Senior Researcher†

I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Name of Senior Researcher† (please print) _____

Signature _____ Date _____

† A senior member of the research team must provide the explanation of, and information concerning, the research project.

Note: All parties signing the consent section must date their own signature.

Appendix D: Sleep and Compliance diaries

Please update this form **every day** while you are participating in this trial.
Please record details of **any problems** that occur, **any medications** that you take (apart from your trial medication) and your **Alertness score** (see Alertness scale **overleaf**). Please take your medication **at least** 12 hours apart at 10am and 10pm. If this isn't possible, please record the times you took the medication in the appropriate spaces below.
Apart from scheduled visits, if at any time before your oocyte collection, you present to a doctor or hospital, please also contact us to let us know (contact details are at the bottom of this page).
Trial ID:_____ Trial medication group (circle): A/B/C/D Monash IVF ID:_____

Day of Trial medication	Date (dd/mm/yy)	Alertness score* (1200)	Morning dose taken (1000)	Evening dose taken (2200)	Details of any undesirable event	Other medications or treatments
Example	***01/01/14***	***2***	***☑Yes ☐No Time: 1010***	***☑Yes ☐No Time: 2210***	***Headache not thought to be related to medication***	***2 tabs Panadol***
1	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
2	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
3	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
4	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
5	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
6	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
7	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
8	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
9	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
10	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
11	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
12	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
13	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		
14	__/__/__		☐Yes ☐No Time:_____	☐Yes ☐No Time:_____		

When you **arrive** for your **egg collection**, **please return this completed form** to the trial coordinator, Dr Shavi Fernando: 95946666 **shavi.fernando@monash.edu**

***Alertness score**

Please use this scoring system to rank your Alertness level **at 12 noon every day** while you are taking the trial medication.

Here are some descriptions about how alert or sleepy you might be feeling right now. Please read them all carefully and write the number that best corresponds to the statement describing how you feel at the moment on your patient diary form.

1. Extremely alert
2. Very alert
3. Alert
4. Rather alert
5. Neither alert nor sleepy
6. Some signs of sleepiness
7. Sleepy, but no difficulty remaining awake
8. Sleepy, some effort to keep alert
9. Extremely sleepy, fighting sleep

MIART - SLEEP DIARY for Actiwatch

Trial ID:_____ Monash IVF ID: _____

Do you **share a bed** with someone for the whole night? Yes/No

Did you consume caffeine (eg. tea, coffee, coke) **in the hour before** bed(circle)? Yes/No **If yes**, how often(circle)? Every night/3-4 nights per week/ 1-2 nights per week

Did you **nap** on any day during this study? Yes/No **If yes**, which days and for how long (list all)?_____

Please record all times to the **nearest 5 minutes**

Day of trial	**Date**	**Time watch taken off**	**Time watch put back on**	**After going to your bedroom what did you do?***	**Time you went to bed**	**Time you went to sleep**	**Number of awakenings during the night**	**Total time awake during the night (mins)**	**Time you woke up next morning**	**What woke you in the morning?****	**Time you got out of bed**	**How well did you sleep last night?*****
Example	***25/5/14***	***7:35pm***	***7:45pm***	***Read a book***	***10:05pm***	***10:20pm***	***1***	***5***	***7:00am***	***Alarm***	***7:10am***	***2***
Nurses visit (Day A)												
B												
C												
D												
E												
F												
G												

Day of trial	**Date**	**Time watch taken off**	**Time watch put back on**	**After going to your bedroom what did you do?***	**Time you went to bed**	**Time you went to sleep**	**Number of awakenings during the night**	**Total time awake during the night (mins)**	**Time you woke up next morning**	**What woke you in the morning?****	**Time you got out of bed**	**How well did you sleep last night?*****
Example	***25/5/14***	***7:35pm***	***7:45pm***	***Read a book***	***10:05pm***	***10:20pm***	***1***	***5***	***7:00am***	***Alarm***	***7:10am***	***2***
Start of trial medication (Day 1)												
2												
3												
4												
5												
6												
7												
8												
9												
10												

11												
12												
13												
14												
15												
16												

*eg. went straight to sleep, watched TV, read a book, used a screen (eg laptop, phone), listened to music, other

**eg. woke myself, a family member, alarm, other

***Please rank from 1 to 4 (1 = very good, 2 = fairly good, 3 = fairly bad, 4 = very bad)

Please make a note of any other activities (with dates and times) which may have effected your sleep below (eg. got sick, stayed out late at a party etc):

Appendix E: Trial medication independent content analysis certificates

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ANALYTICAL REPORT

Company: **Orrong Compounding Pharmacy**

Contact: **Stephanie**

Telephone: [redacted]

Fax: [redacted]

Our reference: **140709-1**

Date: **17/07/14**

Samples:

Melatonin 2 mg capsules Batch# 645312

Raw material used as Reference:

Melatonin Fagron B# 13D09-U03-010769 Purity 99.4% Exp 30/06/15 (RRM-084)

Results:

Test	Method	Average capsule weight	content
Melatonin assay	**SOP AM 129-03**	**312.1 mg**	**1.89 mg/capsule**

Notes: The method has been partially validated.

Analyst: [redacted] Grace Consoni
Book 6 p 161

Reviewed By: chi Hua

Authorised by: [redacted]

Date authorised: 18/07/14

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Australian Life Sciences

ANALYTICAL REPORT

Company: **Orrong Compounding Pharmacy**

Contact: **Stephanie**

Telephone: [redacted]

Fax: [redacted]

Our reference: **140709-2**

Date: **17/07/14**

Samples:

Melatonin 4 mg capsules Batch# 645313

Raw material used as Reference:

Melatonin Fagron B# 13D09-U03-010769 Purity 99.4% Exp 30/06/15 (RRM-084)

Results:

Test	Method	Average capsule weight	content
Melatonin assay	**SOP AM 129-03**	**315.5 mg**	**4.04 mg/capsule**

Notes: The method has been partially validated.

Analyst: [redacted] Grace Consoni
Book 6 p 161

Reviewed By: chi Hua

Authorised by: [redacted]

Date authorised: 18/07/14

Australian Life Sciences
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ANALYTICAL REPORT

Company: **Orrong Compounding Pharmacy**

Contact**: Stephanie**

Telephone: [redacted]

Fax: [redacted]

Our reference: **140709-4**

Date: **17/07/14**

Samples**:**

Melatonin 8 mg capsules Batch# 645314

Raw material used as Reference:

Melatonin Fagron B# 13D09-U03-010769 Purity 99.4% Exp 30/06/15 (RRM-084)

Results**:**

Test	Method	Average capsule weight	content
Melatonin assay	**SOP AM 129-03**	**319.3 mg**	**8.29 mg/capsule**

Notes: The method has been partially validated.

Analyst: [redacted] Grace Consoni
Book 6 p 161

Reviewed By: Chi Hua

Authorised by: [redacted]

Date authorised: 18/07/14

Australian Life Sciences

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